Synthesis of Modified Ribonucleosides and Oligoribonucleotide Hybridization Probes

Thesis by

Michael Robert Lewis

In Partial Fulfillment of the

Requirements for the Degree of

Master of Science

California Institute of Technology

Pasadena, California

1988

(submitted September 29, 1987)

Abstract

Two strategies for the construction of chemically modified, DNA cleaving oligoribonucleotide probes are described. In the first strategy, the synthetic substrate 5-(3-aminoallyl)uridine 5'-triphosphate (AA-UTP, **1**) is reacted with RNA polymerase II to synthesize an aminoallyl-oligoribonucleotide, and the DNA cleaving moiety is introduced by nonspecific, posttranscriptional derivatization of the primary amine groups of the modified RNA. The second strategy involves the incorporation of a modified ribonucleoside 3',5'-bisphosphate, covalently tethered to a DNA cleaving functionality, into an oligoribonucleotide by reaction with T4 RNA ligase. A general method for the chemical synthesis of ribonucleoside 3',5'-bisphosphates was developed. Using this method, uridine 3',5'-bisphosphate (pUp, **2**) was synthesized in six steps with an overall yield of 14%. A modified pUp derivative, 5-(3-(3-nitrophenoxy)acetamidoallyl)-uridine 3',5'-bisphosphate (NA-pUp, **12**), was also synthesized with an overall yield of 3% for nine steps. The RNA polymerase substrate **1** was prepared by known methods and tested for compatibility with T7 RNA polymerase II in an *in vitro* "run-off" transcription system. The incorporation of AA-UTP by T7 RNA polymerase was found to be less efficient than that of uridine 5'-triphosphate (UTP), in contrast to results reported for SP6 RNA polymerase. *In vitro* transcription synthesis of oligoribonucleotides and aminoallyl-oligoribonucleotides by SP6 RNA polymerase gave irreproducible results. In the T7 RNA polymerase system, transcription synthesis produced heterogeneous mixtures of oligoribonucleotides or aminoallyl-oligoribonucleotides. Analysis of the transcription products by high-resolution gel electrophoresis showed that the distribution of transcripts did not vary significantly with buffer conditions or template end structure.

Table of Contents

INTRODUCTION

Incorporation of a reactive chemical moiety into an oligoribonucleotide could provide a molecule capable of cleaving intact, native double helical DNA with great specificity and flexibility of target sequence. This thesis describes the synthesis of modified ribonucleoside phosphates and modified oligoribonucleotides designed for the construction of RNA probes equipped with DNA cleaving functionalities.

Hybridization Probes

Natural and synthetic oligo- and polynucleotides have proven useful for many biological and medical applications in the study of infectious diseases, genetic mutations, recombinant DNA, gene expression, and nucleic acid structure and function. These nucleic acid probes employ a basic biological principle: that a single-stranded nucleotide recognizes and binds to its complementary sequence, a process called hybridization. Such complementary recognition allows oligonucleotide probes to possess high affinity and base-sequence specificity in nucleic acid binding, and chemical modification of these probes provides the opportunity to augment or alter their biological activities.

Chemically modified hybridization probes have been employed in the biophysical study and isolation of nucleic acids. Oligodeoxynucleotide probes covalently linked to nonspecific intercalating agents have been synthesized and characterized recently,[1-4] and the interaction of these probes with complementary sequences of DNA and RNA is strongly stabilized by the intercalator. The supplementary energy of interaction provided by the intercalating agent gives the oligonucleotides great affinity for the target nucleic acid sequence, and such probes complementary to RNA polymerase promoter sites or mRNA sequences have been shown to perturb gene expression *in vivo*.[5] Novel nucleic acid affinity probes have been created by the chemical and enzymatic attachment of biotin to DNA and RNA polynucleotides[6,7] and to tRNA.[8-10]

Biotin-labeled nucleotides can be retained selectively on avidin-Sepharose, immunoprecipitated in the presence of antibiotin antibody, or visualized by electron microscopy with avidin-ferritin crosslinked complex, making these affinity probes useful for the isolation of specific DNA and RNA sequences or the mapping of genetic topography. Fluorescent reporter groups and photoaffinity labels have also been introduced to hybridization probes by both chemical and enzymatic methods. Modification of DNA fragments,[11] polyribonucleotides,[12-15] tRNA,[16,17] 5S rRNA,[17] and oligoribonucleotides[17] with photoactive moieties has created new tools for the study of nucleic acid structure, function, and location.

The synthesis of probes with novel biophysical properties has been paralleled by the development of oligonucleotides equipped with chemically reactive functionalities. Sequence-specific alkylation of both RNA and DNA has been achieved by the use of complementary DNA[18,19] and RNA[20-22] nucleotides, respectively, possessing covalently attached electrophiles. Oligomers of DNA singly labeled with ^{125}I effect radiochemical cleavage of the opposite strand over a range of 15-70 Å from the decay site.[23] Recently, DNA hybridization probes containing the metal chelator EDTA have been synthesized,[24,25] and in the presence of O_2, Fe(II), and dithiothreitol, these DNA-EDTA probes afford specific oxidative cleavage of the complementary sequences over a range of approximately 17 nucleotides, presumably caused by a diffusible reactive species such as hydroxyl radical. While DNA-EDTA oligonucleotides provide a high degree of design specificity in cleaving single-stranded nucleic acid, heterogeneous DNA-DNA hybridizations have not been stabilized in such a way as to allow these probes to react with duplex DNA. However, RNA-DNA interactions have been manipulated to effect *in situ* hybridization to target sequences of double helical DNA, and synthesis of a ribonucleotide equipped with EDTA-Fe(II) or another reactive functional group might produce a probe capable of cleaving its complementary sequence within a region of native double-stranded DNA.

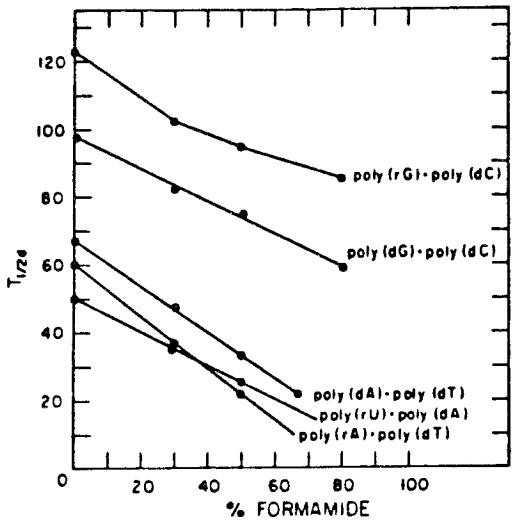
RNA-DNA Interactions

Segments of RNA hybridized to complementary sequences of DNA in a double helical structure were first isolated by equilibrium centrifugation in CsCl gradients in 1961.[26] Since this initial discovery, duplex RNA-DNA hybrids have been assayed quantitatively by immobilization on nitrocellulose membrane filters[27] and detected in cytological preparations after acetic acid squashing.[28,29] The structures of RNA-DNA duplexes have been elucidated by X-ray fiber diffraction and crystallographic techniques and shown to be almost identical to A form DNA.[30,31] In this double-stranded structure, there are 11 residues per helical turn, and a change in the ribose sugar pucker to the C-3 *endo* conformation tilts the base pairs 70-80° to the helix axis. In G-C rich RNA-DNA hybrids, the conformation is partly determined by bridging H_2O molecules between the 2'-hydroxyl group of the ribose and O-2 of cytosine,[31] and since this conformation does not change significantly with the relative humidity of the fiber preparation, it is likely that RNA-DNA duplexes adopt the A form in solution.[30] Fiber diffraction studies of poly(rA)-poly(dT) hybrids, however, reveal the formation of of two conformations depending on the humidity of the sample preparations.[32] At 79% relative humidity, the A-T rich hybrid adopts a conformation like that of A' duplex RNA,[33] while under highly solvated conditions, the conformation is very similar to B form DNA, which has 10 residues per turn of the helix and base pairs perpendicular to the helical axis. A structural model for B-like solution forms of RNA-DNA hybrids was proposed in which the 2'-hydroxyl groups are stabilized in the *endo* conformation by intrastrand hydrogen bonds to adjacent ribose moieties.[32]

In aqueous solution, the thermal stability of RNA-DNA hybirds is intermediate between those of RNA-RNA and DNA-DNA duplexes. RNA-DNA duplex structures have been stabilized in solution by the use of aqueous formamide, since nucleic acid association reactions proceed at much

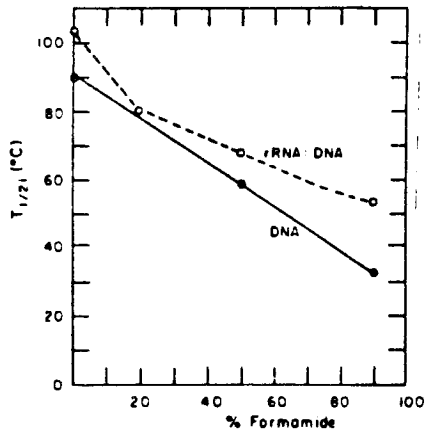
lower temperatures in this solvent than in pure aqueous solutions.[34-37] The thermal stability of DNA-DNA duplexes decreases linearly with increasing formamide concentration,[35] but the stability of RNA-DNA hybrids in high formamide solvents varies with the base composition of the nucleotides.[38] The thermal melting transitions of poly(rA)-poly(dT) and poly(rU)-poly(dA) duplexes occur at slightly lower temperatures than those of poly(dA)-poly(dT) and also decrease linearly with increasing formamide concentration (Figure 1a). The corresponding melting transitions for poly(rG)-poly(dC), however, occur at higher temperatures than those of the analogous DNA-DNA duplex and the melting curve is concave upwards as a function of formamide concentration. If there is intrastrand base composition heterogeneity, as in the case of native nucleic acids, the thermal stability of the duplex molecule is governed by its G-C content, perhaps by a segment of 50-100 nucleotides which has the highest local concentration of G-C base pairs.. Native heterogeneous RNA-DNA hybrids, therefore, behave much like poly(rG)-poly(dC) in high formamide solvents (Figure 1b). Thus, RNA-DNA hybrids are generally more stable than DNA-DNA hybrids in high formamide solvents, and this difference is greatest for G-C rich polynucleotides. At a temperature intermediate between the strand dissociation temperatures of DNA-DNA and RNA-RNA duplexes, the RNA-DNA association reaction occurs almost quantitatively in high formamide, whereas there is no DNA-DNA reaction. Similar stabilization of RNA-DNA hybrids under conditions of DNA duplex denaturation has also been achieved in pure aqueous media by the use of sodium perchlorate, sodium perchlorate-urea, and rubidium trichloroacetate solutions.[39]

An important discovery in the study of RNA-DNA interactions was made when a mixture of single-stranded RNA and longer duplex DNA containing a sequence complementary to the RNA was incubated in high formamide buffer at a temperature just below the denaturation temperature of the DNA. Under these conditions, the RNA displaces its identical DNA segment and forms a



The effect of increasing concentration of formamide on the $T_{1/2d}$'s of various synthetic duplex polynucleotides. The $T_{1/2d}$ measurements were obtained in 0.3 M NaCl, 10 mM Tris pH 7.8 for poly rU:dA and poly dA:dT. Remaining data taken from Fig. 4. All $T_{1/2d}$ measurements are normalized to 0.1 M NaCl (15).

Fig. 1a



The $T_{1/2i}$'s of E. coli DNA and rRNA:DNA hybrids are shown as a function of formamide concentration in 0.3 M NaCl, 0.01 M Tris, pH 7.8. Approximately 5μg of E. coli ^{32}P DNA (specific activity 2000 cpm/μg) was used to determine the $T_{1/2i}$ for each melting curve. The $T_{1/2i}$'s of rRNA:DNA hybrids were determined with 2500 cpm for each melting curve.

Fig. 1b

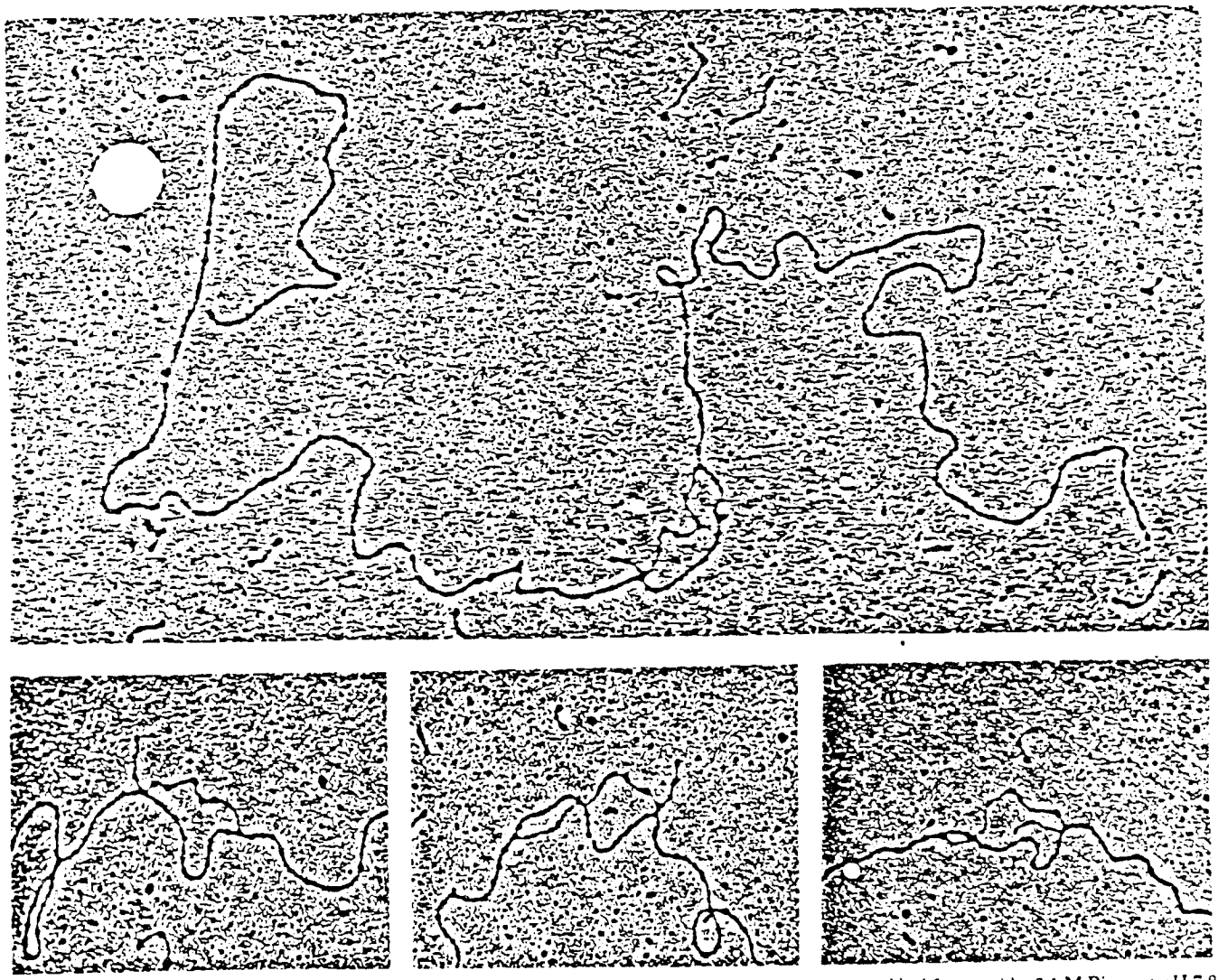
Figure 1. Thermal stabilities of RNA-DNA hybrid duplexes in high formamide buffers.[38]

duplex with the complementary segment, a structure known as an R-loop (Figure 2).[40] In 70% formamide, this process occurs without DNA reannealing at temperatures permitting the optimum rate of R-loop formation.[40,41] The use of a molar excess of RNA allows for formation of a uniform population of R-loops, and large structures are stable for several days at 5°C. Upon removal of formamide, R-loops are thermodynamically unstable and are displaced by branch migration,[42] but this process is sufficiently slow as to render R-loops partially stable to dialysis and heating.[40] R-loops are quite sensitive to RNase A but resistant to DNA cleavage by restriction endonucleases such as EcoRI.[40]

Since the discovery of R-loop formation, the technique has been used for a variety of biological applications, including mapping of genetic topography and transcription products,[43-51] screening of bacteriophage hybrid plaques for inserted DNA sequences,[52] determination of cellular RNA concentrations,[53] enrichment of specific genes,[54-57] and purification of mRNAs.[58,59] R-loop formation could also be used to achieve the sequence-specific cleavage of double helical DNA in intact native substrates if an appropriate ribonucleotide were modified with a chemical functionality capable of reacting with DNA (Scheme I). Such a molecule would bind to a complementary segment of supercoiled DNA in high formamide solvent to produce a circular complex containing an R-loop. Subsequent activation of the reactive functionality should then effect cleavage of the opposite strand of DNA.

Probe Construction

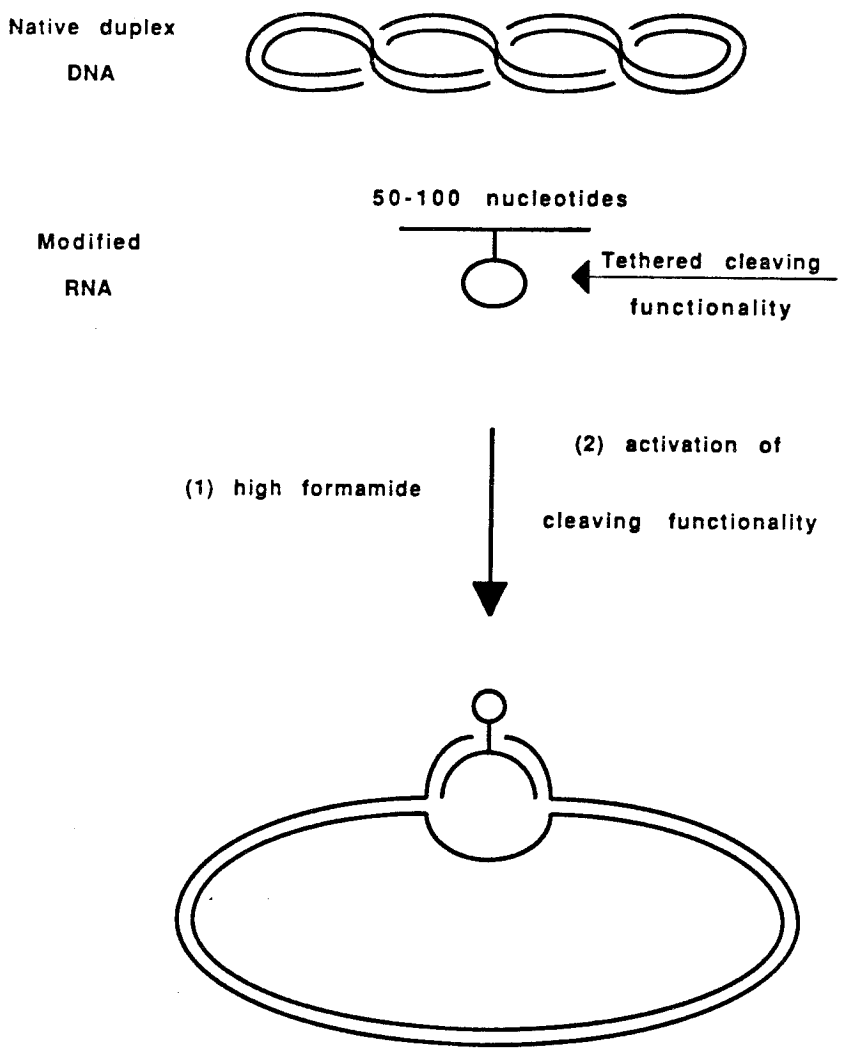
The synthesis of a ribonucleotide probe tethered to a DNA cleaving functionality could be accomplished by chemical methods, enzymatic methods, or a combination of both. By analogy to the synthesis of DNA-EDTA probes,[24] the construction of reactive RNA probes would involve the



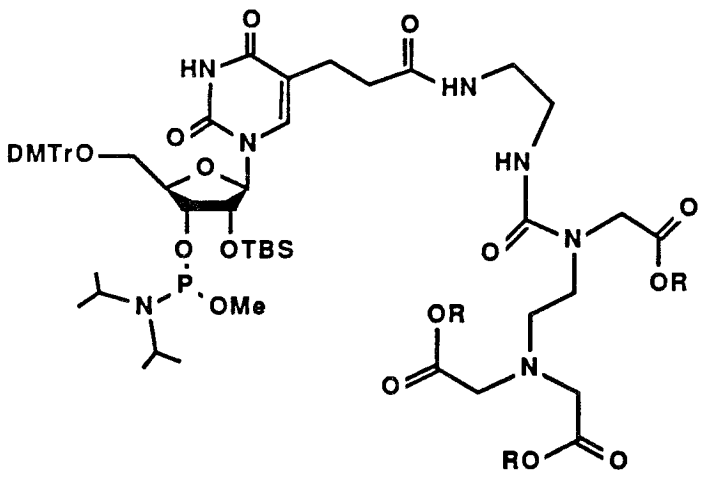
R-loops were made by heating 5 μg/ml of λgt-Sc1109 DNA and 5 μg/ml total rRNA in 70% vol/vol formamide, 0.1 M Pipes at pH 7.8, and 0.01 M Na₃EDTA at 47° for 20 hr. The reaction was performed under oil in a sealed, siliconized glass tube. All 500 RNA molecules examined contained an R-loop similar to those shown. The sample was mounted for electron microscopy by the formamide technique (2). Grids were stained with uranyl acetate and shadowed with Pt/Pd.

Figure 2. Electron micrographs of R-loop structures formed between single-stranded RNA and complementary duplex DNA.[40]

SCHEME I


Native duplex
DNA
50-100 nucleotides
Modified
RNA
Tethered cleaving
functionality
(1) high formamide
(2) activation of
cleaving functionality

preparation of a key ribonucleoside functionalized with EDTA and equipped for solid-phase oligonucleotide synthesis:

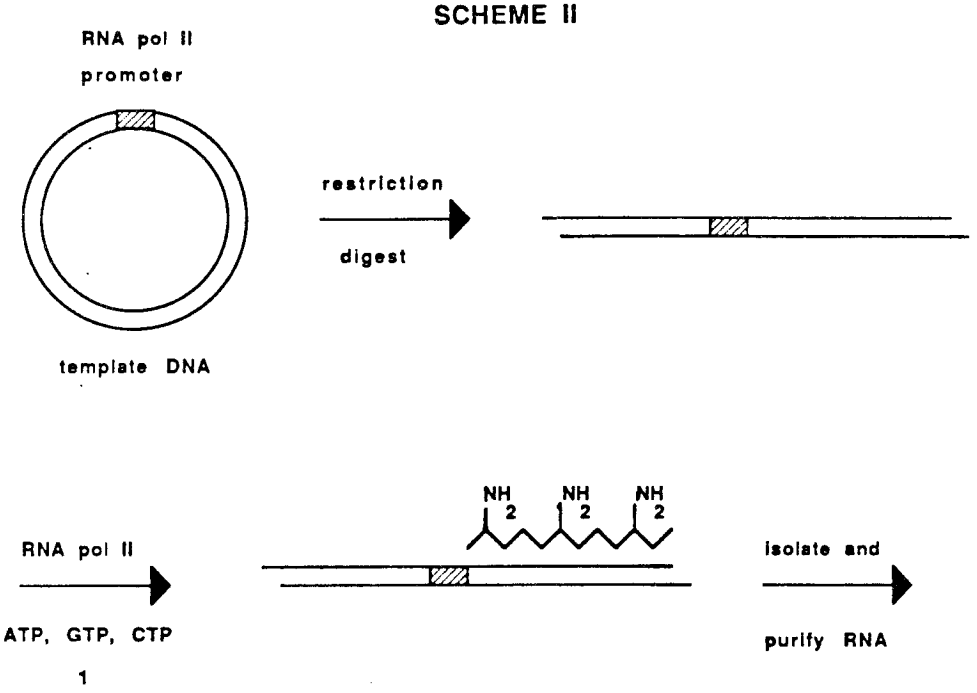


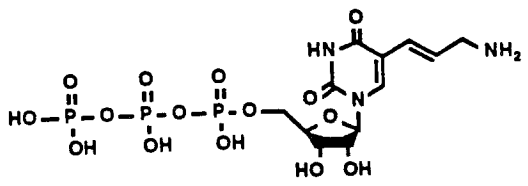
where R is an EDTA protecting group compatible with RNA stability, and the 4,4'-dimethoxytrityl (DMTr) and *tert*-butyldimethylsilyl (TBS) ether groups are required for proper coupling of the nucleosides during solid-phase RNA synthesis. This key nucleoside could then be incorporated into a chemically synthesized oligoribonucleotide at the desired position by solid-phase RNA synthesis techniques.[60-62] However, there are inherent difficulties in this synthetic strategy. First, manual RNA synthesis is considerably more complicated than DNA synthesis and is not yet performed routinely in synthetic laboratories. Furthermore, R-loop formation introduces destabilizing end effects into the RNA-DNA duplex, and short RNAs are rapidly displaced by branch migration.[40] Thus, RNA probes 50-100 nucleotides long, perhaps, may be required for the formation of stable R-loops. Chemical synthesis of ribonucleotides of such great length would be impractical, if not impossible, using existing technology.

Because the chemical synthesis of these RNA probes is rather impractical, enzymatic synthesis of ribonucleotides, followed by enzymatic or chemical incorporation of DNA cleaving moieties, was considered as an alternative strategy for the construction of the desired modified RNAs. *In vitro* transcription by RNA polymerase II is a relatively straightforward, efficient process by which preparation of oligoribonucleotides might be expanded to a "synthetic" scale. Recently, plasmid vectors containing dual promoter sites for SP6[63] and T7[64,65] RNA polymerases II have become comercially available, and on the basis of this consideration, it was decided to explore the scope and limitations of *in vitro* transcription synthesis of oligoribonucleotides by these two enzymes. RNA of uniform length can be transcribed from DNA segments ligated onto these vectors by cleaving the template at a unique termination site for the subsequent RNA polymerase reaction.[63-65] Such RNA transcripts have been employed as probes for *in situ* hybridization to target sequences of both DNA and RNA.[66-68] By this method, RNA probes of the length required for R-loop formation and cleavage of complementary sequences of DNA can be synthesized rapidly and routinely.

With transcription synthesis of an appropriate oligoribonucleotide, attachment of EDTA or any of a number of potential DNA cleaving functional groups to the RNA was considered according to two strategies for probe construction. The first strategy, depicted in Scheme II, involves *in vitro* transcription by RNA polymerase II, using the synthetic substrate 5-(3-aminoallyl)uridine 5'-triphosphate[6,7] (AA-UTP, **1**) to produce an aminoallyl-modified oligoribonucleotide. The DNA cleaving functionality is then introduced by nonspecific, posttranscriptional derivatization of the primary amine groups of the modified RNA. This strategy has the advantage that RNA molecules equipped with potential DNA cleaving groups may be constructed in a two-step process based on existing methodology. While this approach does not permit precise control of the positions where cleaving groups are tethered to the ribonucleotide chain, modified RNA probes of

SCHEME II


RNA pol II
promoter
restriction
digest
template DNA
RNA pol II
ATP, GTP, CTP
1
NH2
NH2
NH2
Isolate and
purify RNA
RCOOH
DCC, NHS
R
R


HO
OH
HO OH
HN
NH2

1

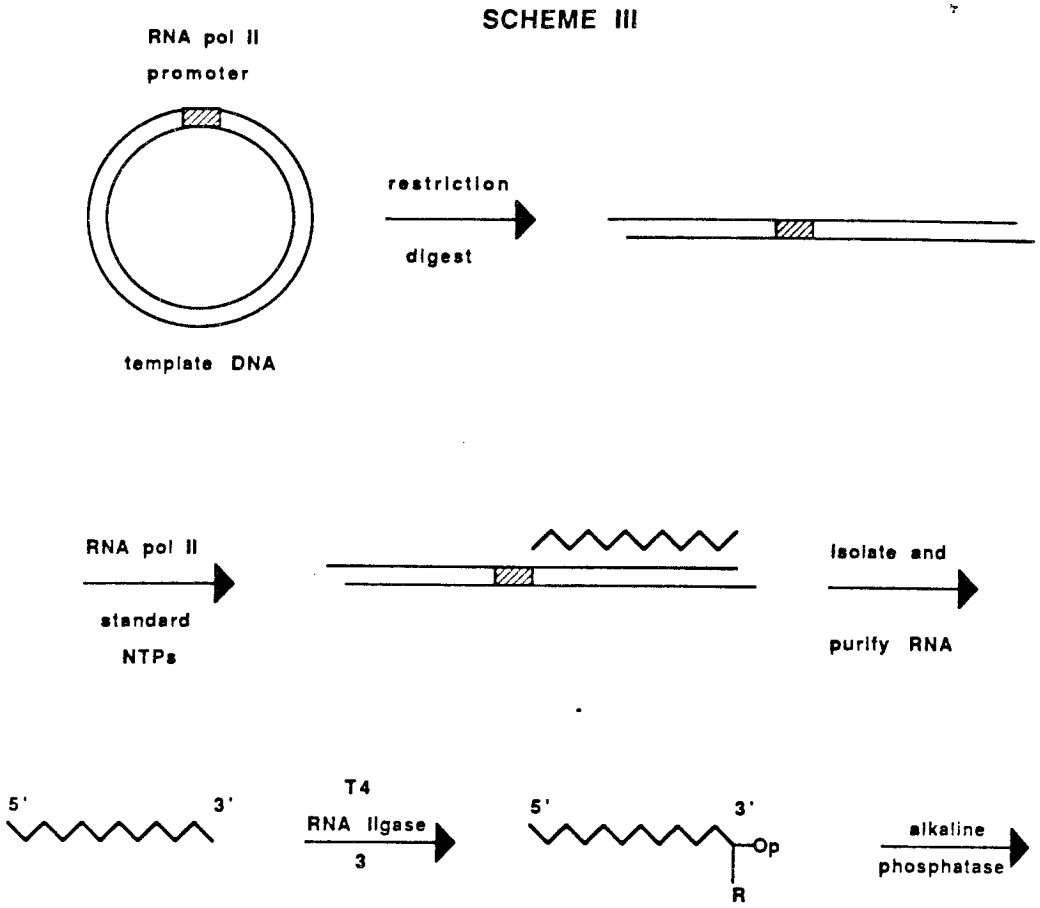
this nature might prove to be useful research tools in the cleavage and separation of large segments of DNA, such as genes.

Specific introduction of a single cleaving functionality to an oligoribonucleotide might be accomplished using a refinement of this strategy. The synthetic substrate **1** can be modified with a carboxylic acid derivative of biotin to produce an amide product which is also active as a substrate for SP6 RNA polymerase II.[6,7] In like manner, **1** could be tethered to a cleaving group by amide bond formation to form a new pseudosubstrate for RNA polymerase. Using a template coding for a single uridine residue in its sequence, the modified nucleoside might be incorporated by the enzyme into a unique position of the transcript sequence.

Another approach to the incorporation of a single DNA-cleaving moiety into an oligoribonucleotide is shown in Scheme III. This second strategy involves the incorporation of a modified ribonucleoside 3',5'-bisphosphate (**3**), covalently tethered to a DNA cleaving moiety, into an unmodified oligoribonucleotide by reaction with T4 RNA ligase.[69] Subsequent reaction with alkaline phosphatase and RNA ligase-mediated coupling with a second oligoribonucleotide,[70] also shown in Scheme III, would afford an RNA probe with a single DNA cleaving functionality in an internal position of the nucleotide sequence. Probe construction with RNA ligase allows for exact placement of the cleaving functionality in the oligoribonucleotide sequence, creating probes which should cleave DNA at a unique site within the complementary sequence.

T4 RNA ligase possesses great potential for the addition of modified nucleosides to RNA molecules. The enzyme catalyzes the formation of a phosphodiester bond between the 5'-terminal phosphate and 3'-terminal hydroxyl groups of oligo- or polynucleotides with the accompanying hydrolysis of ATP.[71-73] While the biological function of RNA ligase is still unclear, the reactions catalyzed by the enzyme have been well characterized and employed for a variety of nucleic acid manipulations. T4 RNA ligase catalyzes the cyclization of sufficiently long RNAs with the

SCHEME III


RNA pol II
promoter
template DNA
restriction
digest
RNA pol II
standard
NTPs
isolate and
purify RNA
5'
3'
T4
RNA ligase
3
Op
R
alkaline
phosphatase
OH
Op
HN
NH
HO-P-O
OH
N
O=P-OH

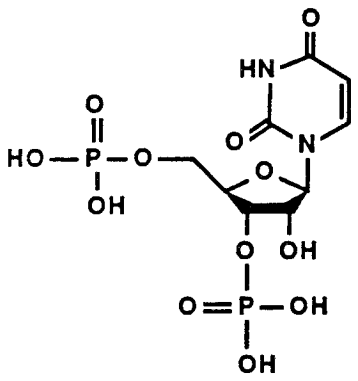
3

appropriate termini,[74] mediates the intermolecular coupling of RNAs,[75] DNAs,[76,77] and block copolymers,[78-80] and stimulates the blunt-end joining of duplex DNA in the presence of T4 DNA ligase.[81]

The mechanism of RNA ligase catalysis is a three-step process.[82] First, hydrolysis of ATP produces pyrophosphate and adenylated enzyme. Then AMP is transferred to the 5'-phosphate of the donor nucleotide to give an intermediate of the form A5'pp5'X. This intermediate has been isolated and shown to be reactive toward acceptor nucleotides in an ATP-independent ligation catalyzed by the enzyme,[83-85] but both the donor and acceptor must be present for the intermediate to be formed.[80] The third step in the ligation mechanism is the nucleophilic displacement of AMP from the intermediate by the 3'-hydroxyl group of the acceptor nucleotide, forming a donor-acceptor internucleotide bond. To accommodate this mechanism, RNA ligase has three binding sites: an acceptor site which requires trinucleotide bisphosphates as minimal substrates,[70] a donor site requiring nucleoside 3',5'-bisphosphates as minimal substrates,[70,86] and an ATP binding site. The enzyme shows little base sequence specificity for donor and acceptor nucleotides,[87] but the ATP binding site is very specific; dATP is the only pseudosubstrate capable of adenylating the enzyme and does so poorly.[88] Because of the low sequence specificity of RNA ligase toward donor and acceptor nucleotides, the enzyme has been used for the synthesis of a variety of heterogeneous oligoribonucleotides,[89-97] as well as the specific 3'-end labeling of RNAs with nucleoside 3',5'-bisphosphates.[69,86,98-100]

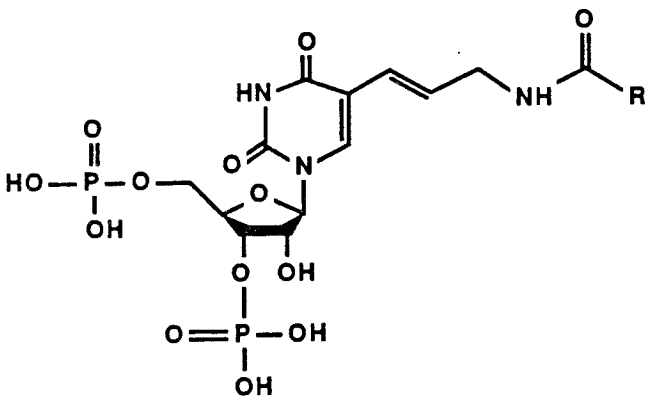
The 3'-end labeling reaction catalyzed by T4 RNA ligase has proven to be a useful method for the incorporation of reporter nucleosides into many different RNAs. Radiolabeling of RNA molecules at the 3'-terminus has been performed with [5'-^{32}P]-cytidine 3',5'-bisphosphate.[99] Since RNA ligase shows very little specificity toward donors of the type A5'pp5'X in the ATP-independent reaction,[85] it is probable that base-substituted nucleoside 3',5'-bisphosphates

could be added to oligoribonucleotides with efficiencies comparable to those of naturally occurring nucleosides. Indeed, several base-modified and sugar-modified nucleosides containing fluorescent moieties have been ligated onto RNAs with the enzyme.[16,17,100] In two cases, stepwise synthesis with RNA ligase and alkaline phosphatase[70] produced RNA molecules with modified nucleosides in internal positions.[100] The flexibility with which RNA ligase incorporates modified nucleosides into RNA molecules invites the encouraging possibility that nucleoside 3',5'-bisphosphates covalently tethered to DNA cleaving functionalities might also be attached to RNA probes in like fashion. Thus, a good donor nucleoside such as uridine 3',5'-bisphosphate (pUp, **2**)



2

might be converted to a modified nucleoside of the general structure **3**,



3

where R is EDTA or perhaps any one of a number of highly reactive organic functional groups potentially capable of reacting with DNA. Ligation of **3** to a ribonucleotide obtained from *in vitro* transcription synthesis would then produce an RNA probe designed for the sequence-specific cleavage of intact, native double helical DNA. As depicted in Scheme III, the cleaving functionality could be placed at the 3'-end of the RNA probe or at a specific internal position of the oligoribonucleotide by this method of probe construction.

RESULTS

AND

DISCUSSION

The construction of DNA-cleaving RNA probes by enzymatic methods called for the preparation of some base-modified derivative of a mononucleoside phosphate, a synthetic substrate to be incorporated into an oligoribonucleotide by an enzyme which catalyzes a particular RNA reaction. Two enzymes which possess great potential for the incorporation of modified nucleosides into RNA molecules are T4 RNA ligase and RNA polymerase II. Thus, synthetic efforts were directed toward two classes of target molecules which would possess both activity as enzyme substrates and capacity for carrying a DNA-cleaving functional group. Uridine 3',5'-bisphosphate (**2**) was synthesized by a new general route to mononucleoside 3',5'-bisphosphates and further modified with a possible DNA-cleaving functional group to produce a potential substrate for T4 RNA ligase.[69] The substrate for RNA polymerase II, 5-(3-aminoallyl)uridine 5'-triphosphate[6,7] (**1**), was synthesized and used in the preparation of aminoallyl-modified oligoribonucleotide probes by *in vitro* transcription synthesis.[63-65]

Synthesis and Modification of Uridine 3',5'-Bisphosphate (2)

Construction of modified oligoribonucleotides with T4 RNA ligase necessitated the preparation of potential RNA ligase substrates of the general structure **3**. Previous chemical syntheses of nucleoside bisphosphates have produced nearly equimolar mixtures of the 3',5'- and 2',5'-isomers,[100-106] but these mixtures are RNA ligase competent because the 2',5'-isomer is neither a substrate nor an inhibitor of the enzyme.[70] However, such mixtures are undesirable for subsequent chemical modification of nucleoside bisphosphates with tethered DNA-cleaving moieties. The modified nucleosides synthesized for the 3'-end labeling of RNA[100] were prepared by modification of the base or ribosylation of a modified base prior to phosphorylation of the nucleoside, but these methods still afford a mixture of isomers and would be impractical for the

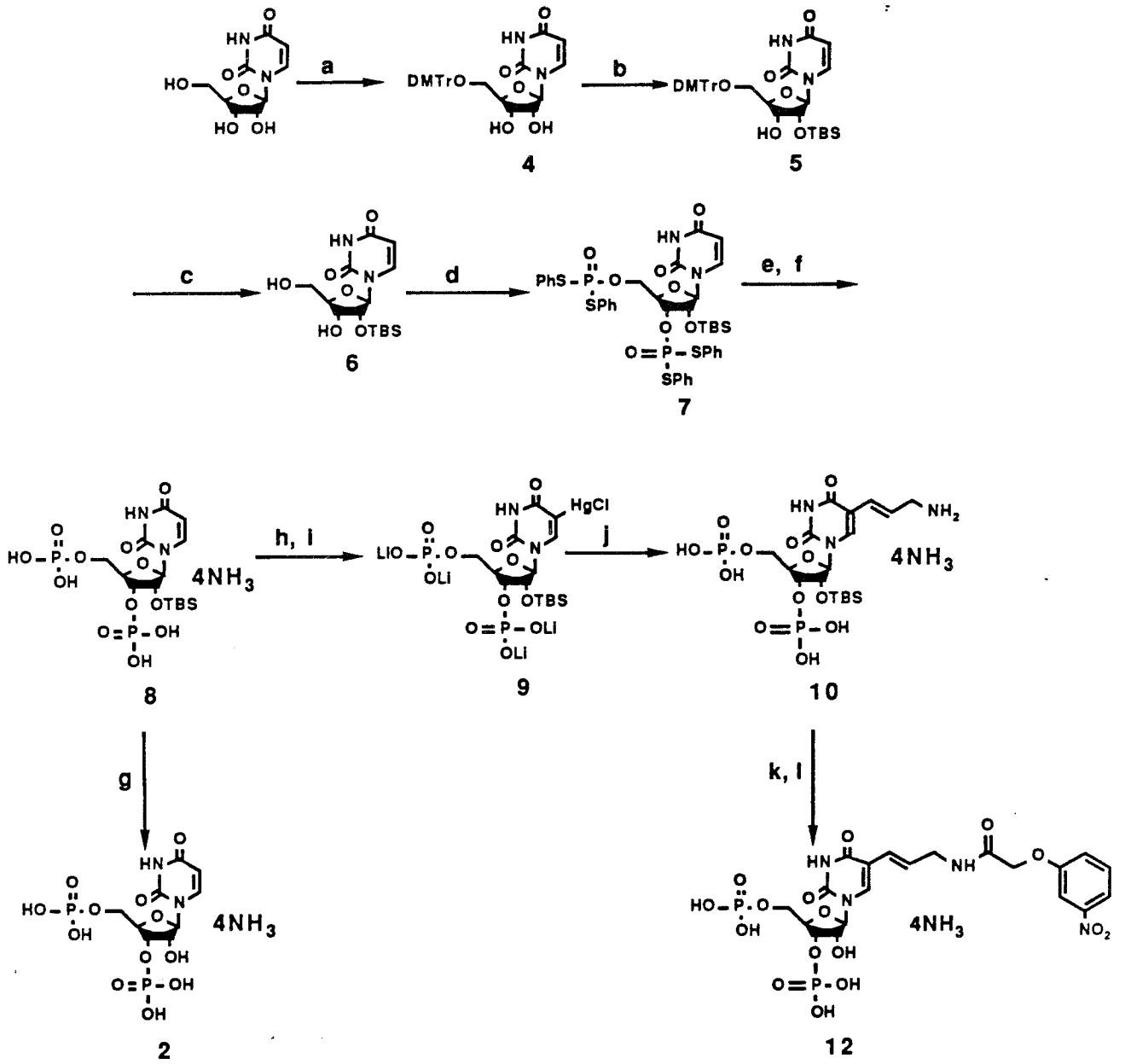
production of a homologous series of modified nucleosides. It was decided to develop a general synthetic scheme in which ribonucleoside 3',5'-bisphosphates modified with a wide variety of tethered DNA-cleaving functionalities could be generated from a common intermediate.

Pure nucleoside 3',5'-bisphosphates have been prepared in moderate amounts by several different enzymatic degradations and phosphorylations,[107-111] but these techniques are limited by the availability of nucleotides in RNA and the specificities of the enzymes. Enzymatic preparations of nucleoside 3',5'-bisphosphates have made commercially available a few of these compounds in modest quantity, sufficient for small-scale organic synthesis. Thus, the initial attempt to synthesize the target nucleoside **3** focused on the attachment of reactive functional groups to the commercially available nucleoside uridine 3',5'-bisphosphate (pUp, **2**).

Uridine can be modified at C-5 by mercuriation,[112] and the resulting mercuriated nucleoside can be coupled to olefins in the presence of Pd(II).[6,112-114] This reaction sequence was attempted on commercially available pUp in order to attach allylamine at C-5,[6] but a mixture of products was obtained. The starting material was analyzed by high-resolution proton and phosphorus NMR and by anion-exchange HPLC, and these analyses revealed the commercially available pUp to be a mixture of two nucleoside phosphates present in a ratio of 42:58. The impurity in the commercial preparation of pUp was determined to be uridine 5'-monophosphate-2',3'-cyclic phosphate, a precursor for the enzymatic synthesis of the 3',5'-bisphosphate, and it comprises 58% of the mixture of nucleosides. Because of the possible difficulties of purifying sufficient quantities of the commercial pUp, it was decided to develop a method for the chemical synthesis of pure uridine 3',5'-bisphosphate (**2**) and pUp derivatives (**3**).

The synthetic route to **2** is shown in Scheme IV. Protection of uridine at the 5'-position was accomplished in 93% yield with dimethoxytrityl (DMTr) chloride.[115] The tritylated product, **4**, was then silylated regioselectively with *tert*-butyldimethylsilyl (TBS) chloride in the presence of

SCHEME IV



a. $DMTrCl$, $AgNO_3$
b. $TBSCl$, $AgNO_3$
c. $C_6H_5SO_3H$
d. $C_6H_{11}NH_3^+$ $(PhS)_2PO_2^-$, MDSCl
e. AgOAc
f. H_2S
g. TBAF

h. $Hg(OAc)_2$
I. LiCl
j. $C_3H_5NH_2$, K_2PdCl_4
k. m-$(NO_2)C_6H_4OCH_2CO_2H$, DCC, NHS
l. TBAF

silver nitrate catalyst[115] to give the 2',5'-diprotected nucleoside **5** in 45% yield. Detritylation with 2% benzenesulfonic acid in chloroform-methanol[116] afforded 90% of 2'-*O*-(*tert*-butyldimethylsilyl)uridine (**6**). This reaction sequence produced a monoprotected nucleoside which could be phosphorylated in the correct positions to give the desired nucleoside 3',5'-bisphosphate.

An attempt at direct phosphorylation of **6** with pyrophosphoryl chloride[100] was then made, but this reaction afforded a mixture of the same two nucleosides present in the commercial pUp, with the composition enriched in the bisphosphate by a ratio of 62:38. Pyrophosphoryl chloride was not a good choice for a phosphorylation reagent because of the unpredictable nature of its reactions with nucleosides: various solvent and temperature conditions have produced nucleoside 2'(3'),5'-bisphosphates,[100-104] nucleoside 5'-monophosphates,[102,117,118] nucleoside 5'-monophosphate-2',3'-cyclic phosphates,[119] and highly phosphorylated nucleosides[120] with the reagent. Undesirably, the hydrochloric acid generated as a by-product in the pyrophosphoryl chloride reaction removed the TBS protecting group from compound **6** and caused cyclization of the 3'-phosphate group to a large degree.

Since the use of pyrophosphoryl chloride did not accomplish the desired phosphorylation of nucleoside **6**, an alternate phosphorylation scheme was developed with the use of *S*,*S*-diphenylphosphorodithioate (PSS)[121,122] to introduce protected phosphate groups onto the nucleoside. This reagent could be prepared easily from methyl dichlorophosphate[121] in 79% yield and stored dessicated at -20°C for about two weeks before losing activity. Condensation of **6** with PSS, mediated by 2,4-mesitylenedisulfonyl chloride (MDSCl),[122] afforded the desired bisphosphorodithioate **7** in 50% yield. The phosphorodithioester groups were then removed from **7** with silver acetate[123] to give an 83% yield of the 2'-silylated bisphosphate **8**.

Unexpectedly, the preparation of nucleoside **8** proved to be the crucial step in the synthesis of

uridine 3',5'-bisphosphate and its derivatives. Deprotection reactions performed with fresh reagent grade silver acetate worked reasonably well, providing yields of approximately 67%, but when the same silver acetate was used four months later, a nearly intractable mixture of products was obtained. Separation of these two products was achieved by analytical and preparative strong anion-exchange HPLC, and they were identified as uridine 5'-monophosphate-2',3'-cyclic phosphate and the desired 2'-*O*-(*tert*-butyldimethylsilyl)uridine 3',5'-bisphosphate (**8**) on the basis of high resolution proton NMR spectra.

The conditions required to separate these two uridine phosphates were sufficiently harsh as to cause considerable hydrolysis of the semipreparative HPLC column, rendering this separation impractical for the preparation of even milligram quantities of pure product. Therefore, reaction parameters for the silver acetate deprotection were varied extensively in order to establish conditions by which nucleoside **8** could be prepared and purified easily. Finally, the silver acetate was purified stringently[124] to give long, colorless needles of the reagent. This highly purified silver acetate possessed significantly greater activity in removing the phosphorodithioester groups from **7**, and the desired bisphosphate was obtained cleanly and reproducibly in 83% yield. It was also discovered that nucleoside **8** is unstable to lyophilization from unbuffered aqueous media, presumably because carbonic acid (dissolved CO_2) in doubly distilled water causes desilylation and subsequent phosphate cyclization to give a mixture of the same two products obtained with impure silver acetate. Lyophilization from 0.1 *M* ammonium bicarbonate, pH 7.9, solved this problem and allowed pure 2'-silylated bisphosphate to be isolated.

Removal of the TBS group from nucleoside **8** with tetrabutylammonium fluoride (TBAF)[125] yielded 89% of pure uridine 3',5'-bisphosphate (**2**). The preparation of **2** was accomplished in six steps with an overall yield of 14%. This is the first example of the chemical synthesis of a pure nucleoside 3',5'-bisphosphate, which is an important development in that these compounds may

now be synthesized in large quantities by a general procedure. The procedure is also amenable to the preparation of base-modified pUp derivatives (**3**) in which the nucleoside is tethered to potential DNA-cleaving functional groups.

Modification of pUp was accomplished by attachment of a side chain to C-5 of the uracil ring, also depicted in Scheme IV. Nucleoside **8** was converted to its allamine adduct by mercuriation at C-5[6,112] to give the chloromercuriuridine compound **9** in 74% yield, followed by the Pd(II)-mediated coupling of allylamine[6] to afford a 49% yield of 5-(3-aminoallyl)-2'-*O*-(*tert*-butyldimethylsilyl)uridine 3',5'-bisphosphate (**10**). This allylamine adduct of pUp serves as a universal precursor for the covalent modification of the nucleoside, and ultimately the oligoribonucleotides into which it is incorporated, with reactive functionalities. From compound **10**, a two-step reaction sequence involving amide bond coupling[126] and removal of the silyl group[125] can be used to synthesize a number of target compounds of the general structure **3**. This procedure, outlined in Scheme IV, has been employed in the modification of pUp with one potential DNA-cleaving moiety, 3-nitroanisole. The 3-nitroanisole derivative of pUp was prepared by reaction of nucleoside **10** with 1,3-dicyclohexylcarbodiimide (DCC) and the *N*-hydroxysuccinimide (NHS) ester[126] of 3-nitrophenoxyacetic acid[127] to give the amide product **11** in 57% yield, and deprotection of the 2'-hydroxyl group with fluoride[125] produced 5-(3-(3-nitrophenoxy)acetamidoallyl)uridine 3',5'-bisphosphate (NA-pUp, **12**) in 90% yield.

Both 3-nitroanisole and the related compound 4-nitroveratrole have been shown to react with a variety of nucleophiles via an S_N2 ($^3Ar^*$) mechanism.[128-130] Upon irradiation at 313-330 nm, this reaction proceeds from an excited triplet state of these chromophores, and the intermediate σ complex leads to nucleophilic photoaromatic substitution at the position *meta* to the nitro group and sometimes at the *ortho* position. In the reaction of 4-nitroveratrole, *meta* substitution is only observed for hard nucleophiles such as ammonia or methylamine; when the amine increases in

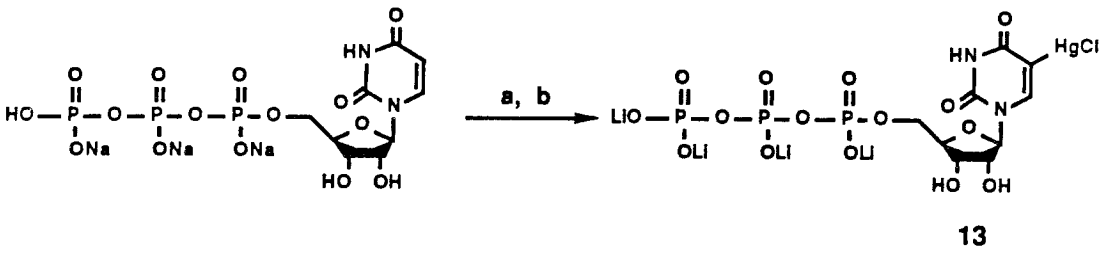
volume and softness, the orientation changes to give preferential *para* attack.[131] 4-Nitroveratrole derivatives of maleimide[132] and an antibiotic[133] have been employed for protein crosslinking and as *in vitro* models for photoaffinity labeling of receptor sites. 4-Nitroveratrole, 3-nitroanisole, and *N*-methyl (3-nitrophenoxy)acetamide have been shown to cleave DNA in a base-specific, nondiffusible manner at guanine residues after visible irradiation.[134] The mechanism of the DNA reaction, however, is not likely nucleophilic photoaromatic substitution, since photolysis of a 1:1 mixture of triacetylguanosine and 3-nitroanisole results in complete destruction of the nucleoside with recovery of the vast majority of the nitroanisole in intact form and no methanol produced.[135]

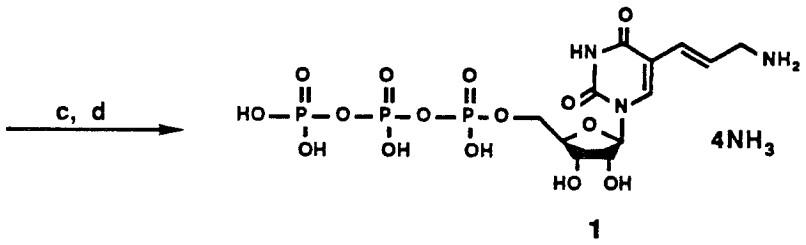
Unfortunately, the incorporation of 3-nitroanisole and 4-nitroveratrole derivatives of 2'-deoxythymidine 5'-triphosphate into oligodeoxyribonucleotides with the large (Klenow) fragment of DNA polymerase I did not produce DNA probes capable of cleaving their complementary sequences photochemically,[136] so it is unlikely that RNAs modified with 3-nitroanisole would be viable DNA-cleaving molecules. The photochemical reaction between these nitroaromatic compounds and DNA may be an electron-transfer process, which is likely to depend dramatically on the distance and orientation of the reactants, and thus the tethering of nitroanisole to a nucleotide may render the chromophore unreactive.

Synthesis of 5-(3-Aminoallyl)uridine 5'-Triphosphate (1)

The synthesis of 5-(3-aminoallyl)uridine 5'-triphosphate (**1**) was considerably shorter and more straightforward than the synthesis of the corresponding uridine 3',5'-bisphosphate derivative (**10**). Compound **1** was prepared by known procedures,[6,7] and this reaction sequence is shown in Scheme V. Reaction of uridine 5'-triphosphate with mercuric acetate, followed by addition of lithium chloride,[6] produced a quantitative yield of 5-chloromercuriuridine 5'-triphosphate, **13**, and

SCHEME V





a. $Hg(OAc)_2$
b. LiCl
c. $C_3H_5NH_2$, K_2PdCl_4
d. strong anion-exchange HPLC

coupling of this aryl anion equivalent to allylamine in the presence of potassium tetrachloropalladate(II)[6] gave, after anion-exchange Sephadex chromatography, a 20% yield of reasonably pure nucleoside **1**.

The purity of 5-(3-aminoallyl)uridine 5'-triphosphate was improved from 89.4% after Sephadex chromatography to 95.8% by strong anion-exchange HPLC, using a volatile buffer system[137] to facilitate isolation of the purified nucleoside. Before HPLC purification, crude **1** contained many nucleoside impurities, including starting material (**13**) and uridine 5'-triphosphate (UTP), produced by reduction of **13** during the palladium coupling reaction. Both of these impurities are competitive inhibitors of the incorporation of **1** into ribonucleic acids by SP6 RNA polymerase II and are therefore undesirable in subsequent *in vitro* transcription synthesis. After HPLC purification of **1**, the only remaining impurities were the aminoallyl derivatives of uridine 5'-diphosphate (2%) and. uridine 5'-monophosphate (2%), neither of which are substrates nor inhibitors in the SP6 system. In fact, these impurities in the final product were anticipated as the HPLC purification of **1** was carried out at pH 9.0, where some hydrolysis of the triphosphate moiety is inevitable.

Synthesis Oligoribonucleotides and Aminoallyl-Oligoribonucleotides

The 4.3kb template chosen for *in vitro* transcription synthesis of oligoribonucleotide hybridization probes contained a 1.37kb fragment of λ phage DNA (positions 26104 to 27479 in the λ genome) cloned into a pUC18-derived vector between the promoter sites for SP6 and T7 RNA polymerases II. This vector, known as the Riboprobe Gemini™ (pGEM™) Positive Control Template, was originally obtained as a gift from Promega Biotec, but it was also grown in much larger quantities in *Escherichia coli* strain HB101 and isolated in supercoiled form by a modification of known procedures.[138,139] Linear templates for "run-off" transcriptions were prepared from

the vector by cleavage of the plasmid with EcoRI and Hinc II restriction endonucleases.[140] Cleavage of the template with EcoRI produced a "run-off" site for the transcription reaction 43 base pairs downstream from the T7 promoter site, while digestion with Hinc II gave a termination site 172 base pairs downstream from the SP6 start site. In order to determine the effect of end structure on the uniformity of the transcripts produced, templates for some T7 transcription reactions were modified after EcoRI digestion by extension of the 3'-end with the large (Klenow) fragment of DNA polymerase I[141] or by degradation of the 5'-end by Nuclease S1.[142] Unmodified oligoribonucleotides were prepared by *in vitro* transcription using standard ribonucleoside triphosphates and the appropriate RNA polymerase. Substitution of 5-(3-aminoallyl)uridine 5'-triphosphate (**1**) for UTP in the RNA polymerase reaction resulted in the synthesis of the corresponding aminoallyl-oligoribonucleotides, in which each uridine residue was modified with an allylamine side chain.

After linearization (and, if appropriate, modification) of the template, numerous transcription assays were performed with SP6[63] and T7[64,65] RNA polymerases II, and comparisons were made between RNA yields obtained with the two enzymes in various buffer systems and with various template end structures. The results of these experiments are summarized in Tables I and II. Table I lists the RNA yields obtained with the T7 and SP6 enzymes in various buffer systems, while Table II describes the effects of template end structure modification on the RNA yield from T7 RNA polymerase. Each value in the tables represents an average yield determined on the basis of 9 to 11 separate transcription assays. It is interesting to note that reasonable and reproducible RNA yields were not obtained with SP6 RNA polymerase II under any of the conditions tried, but generally excellent yields of transcripts from the T7 enzyme could be achieved routinely from the same assay conditions and the same preparations of buffers. Modification of the template end structure with Klenow enzyme did not affect the transcript yield from T7 RNA polymerase II, but

TABLE I

RNA yields (μg RNA/μg template)

Buffer System	T7 pol II	SP6 pol II
A	0.70 ± 0.28	0.02 ± 0.02
B	1.18 ± 0.60	0.05 ± 0.05
C	1.24 ± 0.52	—
D	0.05	—

Buffer systems

A 7.6 m*M* Tris-HCl, pH 7.5, 10 m*M* NaCl, 6 m*M* $MgCl_2$

B 40 m*M* Tris-HCl, pH 7.9, 6 m*M* $MgCl_2$, 2 m*M* spermidine-3HCl

C 40 m*M* Tris-HCl, pH 7.5, 20 m*M* NaCl, 12 m*M* $MgCl_2$, 2 m*M* spermidine-3HCl

D 40 m*M* Tris-HCl, pH 8, 20 m*M* $MgCl_2$

TABLE II

RNA yields (μg RNA/μg template)

End Structure (Template Modification)	T7 pol II
5' overhang (EcoRI)	0.76 ± 0.32
Blunt (Klenow enzyme)	0.76 ± 0.12
Blunt (Nuclease S1)	0.36

degradation of the overhang with Nuclease S1 caused a significant decrease in RNA yield, presumably by the introduction of extraneous nicks in the template.

The control experiments performed with the SP6 and T7 transcription systems suggest that the discrepancy in yield obtained from the two enzymes may be a result of differences in specific activity, concentration, or purity of preparation. The T7 RNA polymerase II (US Biochemical) was obtained from an overproducing clone of phage T7-infected *E. coli*, and the preparations used were extremely pure, active, and concentrated. The preparations of SP6 RNA polymerase II (New England Biolabs and Boehringer Mannheim) employed in the transcription experiments were isolated from phage SP6-infected *Salmonella typhimurium*, and these enzyme preparations are not nearly as pure, active, or concentrated as the T7 enzyme. The SP6 RNA polymerase has an inherently lower specific activity than T7 RNA polymerase, and because the isolated concentration of SP6 enzyme is an order of magnitude lower, not nearly as much SP6 polymerase activity may be utilized in the enzyme-limiting reaction conditions. Unlike the T7 enzyme, some ribonuclease activity also existed in the SP6 preparations, another factor which may have contributed to the lack of sucess with this system. Changes in the reaction conditions and the source of SP6 RNA polymerase need to be explored if this enzyme is to be used effectively for the *in vitro* transcription synthesis of modified oligoribonucleotide hybridization probes.

Figure 3 shows an autoradiogram of ^{32}P-labeled RNA (Lane 1) and aminoallyl-RNA (Lane 2) synthesized by "run-off" transcription with T7 RNA polymerase II and separated on a high-resolution Maxam-Gilbert sequencing gel. The pGEM™ Positive Control Template DNA was linearized with EcoRI restriction endonuclease to produce a termination site 43 base pairs downstream from the T7 start site. The sequence transcribed corresponds to positions 26104 to 26147 on the λ genome, 5'-AAUUCUAAGCGGAGAUCGCCUAGUGAUUUUAAACUAUUG-CUGG-3'. Transcription reactions were performed in buffer system C (40 m*M* Tris-HCl, pH 7.5,

Figure 3. Autoradiogram of transcripts from EcoRI-linearized pGEM™ Positive Control Template DNA produced by T7 RNA polymerase II. Template DNA was incubated with T7 RNA polymerase II and standard mononucleoside triphosphates (lane 1), or with T7 RNA polymerase II and 5-(3-aminoallyl)uridine 5'-triphosphate in place of UTP (lane 2). The product RNAs (lane 1) and aminoallyl-RNAs (lane 2) were uniformly labeled with [α-^{32}P]CTP. After crude purification and desalting on Sephadex G-50-80, labeled nucleotides were diluted to $\leq$1.5 mR $h^{-1}mL^{-1}$, lyophilized, and resuspended in formamide loading buffer. A total activity of ~0.4 mR h^{-1} was loaded in each lane.

20 m*M* NaCl, 12 m*M* $MgCl_2$, and 2 m*M* spermidine-3HCl), using standard ribonucleoside triphosphates to synthesize the unmodified RNA and substituting 5-(3-aminoallyl)uridine 5'-triphosphate (**1**) for UTP in the case of the aminoallyl-oligoribonucleotide, which has an (aminoallyl)uridine residue in place of every uridine in the native RNA sequence. As shown in Figure 3, the positively charged primary amine groups in the modified RNA reduce the migration of the oligoribonucleotide in the electrophoretic system. Yields of aminoallyl-RNA transcript ranged from 40-65% of the yield for corresponding RNA, implying that **1** is less active than UTP as a substrate for T7 RNA polymerase. This finding is particularly interesting considering that reports in the academic and commercial literature state that aminoallyl-UTP is more active than UTP in the SP6 system.

It is evident from the autoradiogram that a heterogeneous mixture of products was obtained in each transcription reaction, affording an ensemble of transcripts varying by about four nucleotides in length. The cause of this phenomenon is that RNA polymerase II melts a "bubble," about 7-12 nucleotides in length, in the duplex structure of the template at the site of polymerization. When this "bubble" is sufficiently close to the end of a linear template, the transcription complex becomes unstable and the enzyme, template and transcript dissociate.[143] The result is that a mixture of RNAs is produced as the complex dissociates at slightly different stages of the polymerization mechanism, creating heterogeneity in the 3'-ends of the transcripts.

Modification of the template end structure from a 5'-overhang to blunt ends with either Klenow fragment or Nuclease S1 did not affect this distribution of transcripts significantly. The mixture of products did not vary much with the different buffer systems employed in the T7 transcription system, either. However, a single band in the autoradiogram accounts for the vast majority, perhaps as much as 90%, of the RNA synthesized in each case, and the discreet oligoribonucleotide or aminoallyl-oligoribonucleotide corresponding to this band could be purified routinely by

preparative polyacrylamide gel electrophoresis.

EXPERIMENTAL

General. Reagent grade chemicals were used without further purification unless otherwise noted. Water for aqueous-phase synthesis, HPLC, and molecular biology was doubly distilled and passed over an activated charcoal filter before use. Dry *N, N*-dimethylformamide (DMF) was stored over activated 4A molecular sieves. Dry pyridine was distilled under argon from *p*-toluenesulfonic acid, then from calcium hydride, and stored over activated 4A molecular sieves. Dry tetrahydrofuran (THF) was freshly distilled under argon from sodium/benzophenone before use. Silver acetate was stirred at room temperature with glacial acetic acid for 24 h, collected by filtration, stirred with fresh glacial acetic acid for 24 h, dried thoroughly *in vacuo*, and twice recrystallized from 0.05% aqueous acetic acid to give long, colorless needles.[124]

Flash chromatography and short column chromatography were performed with Woelm-Pharma silica gel, 32-63 μ particle size. Diethylaminoethyl (DEAE) Sephadex A-25-120 and Sephadex G-50-80 chromatography resins were from Sigma. Thin-layer chromatography was performed with EM Reagents 0.25-mm silica gel 60 F-254 precoated plates. Melting points were recorded on a Thomas-Hoover capillary melting point apparatus and are uncorrected.

Proton NMR spectra were obtained at 90 MHz and 400 MHz on a Varian Associates EM-390 spectrometer and a Jeol GX-400 spectrometer, respectively, using tetramethylsilane (TMS) or solvent absorptions as an internal reference. Chemical shifts are reported relative to TMS. Phosphorus NMR spectra were recorded without proton decoupling on a Bruker WM-500 spectrometer, operating at 202.5 MHz for ^{31}P. Chemical shifts are reported relative to an external standard of 85% H_3PO_4. UV-VIS spectra were recorded on a Cary 219 spectrophotometer. IR spectra were obtained on a Shimadzu IR-435 spectrophotometer and are uncalibrated. HPLC analysis was performed on a Beckman Model 421A HPLC system equipped with Model 114M pumps, Model 165 Variable Wavelength Detector, and a Spectra-Physics SP4290 Integrator.

Solutions of dithiothreitol (DTT) were prepared freshly. Ribonucleoside 5'-triphosphates were

from Pharmacia Biotechnololgies, Inc., and [α-^{32}P]CTP was from Amersham. In addition to being synthesized, 5-(3-aminoallyl)uridine 5'-triphosphate is now commercially available from BRL. EcoRI restriction endonuclease was purchased from Pharmacia, while Hinc II was obtained from New England Biolabs. The large (Klenow) fragment of *E. coli* DNA polymerase I and Nuclease S1 were both from Boehringer Mannheim. RNasin® ribonuclease inhibitor was from Promega Biotec. T7 RNA polymerase II was purchased from US Biochemical Corp., and SP6 RNA polymerase II was obtained from both New England Biolabs and BMB. RNase-free deoxyribonuclease I was purchased from Cooper Biomedical and stored in 0.15 *M* NaCl at -80°C. Riboprobe Gemini™ Positive Control Template DNA was a gift from Promega Biotec.

5'-*O*-(4,4'-Dimethoxytriphenylmethlyl)uridine (4).[115] To a solution of 10.0 g (41.0 mmol) of uridine in 1250 mL of dry THF under an argon atmosphere were added sequentially 16.2 g (16.5 mL, 205 mmol) of dry pyridine, 6.97 g (41.0 mmol) of silver nitrate, and 13.9 g (41.0 mmol) of 4,4'-dimethoxytrityl chloride. The reaction mixture was stirred at room temperature for 1 h, after which it was filtered to remove a granular white precipitate and poured into 1500 mL of 5% $NaHCO_3$. The resulting solution was extracted with 2200 mL of CH_2Cl_2, and the organic layer was dried over Na_2SO_4. The solvent was removed by rotary evaporation to give an orange oil, which was dried *in vacuo* to give 20.8 g (93%) of **4** as a light orange, crispy foam. A sample was recrystallized from Et_2O for the purposes of characterization, affording a pale yellow solid, mp 114-115°C. TLC (MeOH:$CHCl_3$, 1:9): R_f=0.36. ^{1}H NMR ($CDCl_3$): δ 7.98 (1H, d, H6), 7.30 (2H, d), 7.22 (7H, m), 6.76 (4H, d), 5.86 (1H, d, H1'), 5.40 (1H, d, H5), 4.44 (1H, t, H2'), 4.34 (1H, m, H3'), 4.24 (1H, m, H4'), 3.82 (6H, s, OCH_3), 3.52 (2H, m, H5') ppm.

5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(*tert*-butyldimethylsilyl)uridine (5).[115] To a solution of 0.96 g (1.8 mmol) of **4** in 18 mL of dry THF under an argon atmosphere were added sequentially 0.530 g (0.54 mL, 6.70 mmol) of dry pyridine and 0.362 g (2.13 mmol) of silver nitrate. The mixture was stirred for 5 min at room temperature until all the silver nitrate had dissolved, then 0.348 g (2.31 mmol) of *tert*-butyldimethylsilyl chloride was added at once. The reaction mixture was stirred at room temperature for 2.5 h and then filtered to remove a granular gray precipitate. The filtrate was poured into 20 mL of 5% $NaHCO_3$, and the resulting solution was extracted with 65 mL of CH_2Cl_2. The organic phase was dried *in vacuo* to a pale yellow foam, from which the desired product was purified by short column chromatography on silica gel (2 × 6 cm, 10 g silica/g crude product) using Et_2O:hexane (1:1) to give 523.3 mg (45%) of **5** as a light yellow foam, mp 101-103°C. TLC (MeOH:CH_2Cl_2, 5:95): R_f=0.31. ^1H NMR ($CDCl_3$): δ 8.76 (1H,br s, NH), 7.79 (1H, d, H6), 7.10 (9H, m), 6.68 (4H, d), 5.80 (1H, d, H1'), 5.14 (1H, d, H5), 4.23 (2H, m, H2' and H3'), 3.96 (1H, m, H4'), 3.66 (6H, s, OCH_3), 3.36 (2H, m, H5'), 2.44 (1H, d, OH), 0.74 (9H, s, $SiC(CH_3)_3$), 0.02 (3H, s, $SiCH_3$), 0.00 (3H, s, $SiCH_3$) ppm.

2'-*O*-(*tert*-Butyldimethylsilyl)uridine (6).[116] To 3.63 g (5.49 mmol) of **5**, cooled to 0°C, was added 100 mL of a solution containing 2% (2.00 g, 12.6 mmol) of benzenesulfonic acid in $CHCl_3$:MeOH (7:3) with continuous stirring. The reaction mixture was stirred at 0°C for 3 min and immediately poured into 100 mL of 5% $NaHCO_3$. The resulting mixture was extracted with 50 mL of $CHCl_3$. The organic phase was washed with 100 mL of H_2O, dried over Na_2SO_4, and concentrated to dryness by rotary evaporation. The residue was purified by flash chromatography on silica gel (4.5 × 15 cm) using MeOH:CH_2Cl_2 (1:9) to give 1.77 g (90%) of **6** as a pale yellow solid, mp 183-185°C. TLC (MeOH:$CHCl_3$, 1:9): R_f=0.39. ^1H NMR (acetone-d_6): δ 10.06

(1H, br s, NH), 8.04 (1H, d, H6), 5.94 (1H, d, H1'), 5.62 (1H, d, H5), 4.42 (1H, t, H2'), 4.22 (1H, m, H3'), 4.04 (1H, m, H4'), 3.82 (2H, m, H5'), 3.79 (1H, d, OH), 2.86 (1H, s, OH), 0.86 (9H, s, $SiC(CH_3)_3$), 0.12, 0.08 (6H, 2s, $SiCH_3$) ppm.

Attempted Synthesis of 2'-*O*-(*tert*-Butyldimethylsilyl)uridine 3',5'-bisphosphate (8).[100] To 216 mg (0.603 mmol) of **6**, cooled to -20°C in a MeOH-ice bath, was added 1.52 g (0.82 mL, 6.03 mmol) of fresh, chilled pyrophosphoryl chloride with continuous stirring under an argon atmosphere. The temperature of the reaction mixture rose to -5°C and could not be maintained below this temperature with the MeOH-ice bath. The mixture was stirred under argon for 4.5 h at -5°C, and then the reaction was quenched with the rapid addition of ice, followed immediately by the addition of 12 mL of chilled 0.5 *M* ammonium bicarbonate, pH 8.0. The reaction mixture was then concentrated to dryness *in vacuo* at room temperature. The residue was triturated and coevaporated with MeOH three times to remove excess NH_4HCO_3, and it was applied to a DEAE Sephadex A-25-120 column (2 × 15 cm) in 5 mL of 0.05 *M* NH_4HCO_3, pH 8.0. A step gradient, total volume 500 mL, was eluted from 0.05 *M* to 1.0 *M* NH_4HCO_3, pH 8.0, and UV-absorbing fractions were collected at 0.1 *M*, 0.2-0.3 *M*, and 0.4-0.5 *M* NH_4HCO_3. The buffer was removed from these fractions by lyophilization, and each sample was relyophilized from H_2O to a white powder. The fractions collected at 0.1 *M* and at 0.2-0.3 *M* NH_4HCO_3 did not contain the desired product. From the band eluting at 0.4-0.5 *M* NH_4HCO_3, 312 mg of a white powder was obtained which was determined to be a 62:38 mixture of two nucleoside products, uridine 3',5'-bisphosphate (**2**) and uridine 5'-monophosphate-2',3'-cyclic phosphate. ^1H NMR (D_2O): δ 7.82 (1H, d, H6), 7.78 (1H, d, H6), 5.80 (1H, d, H5), 5.79 (1H, d, H5), 5.73 (1H, d, H1'), 5.70 (1H, d, H1'), 4.40 (m, H4'), 4.34 (m, H4'), 4.20 (1H, t, H2'), 4.16 (2H, m, H3'), 4.01 (1H, m, H2'), 3.74 (4H, m, H5') ppm. ^{31}P NMR

(D_2O): δ 3.56 (d, P3'), 3.39 (m, P5'), 3.35 (s), 3.30 (s), 2.04 (s), 1.17 (m), -7.53 (s) ppm.

***S,S*-Diphenylphosphorodithioate.**[121] To 22.5 mL of dry pyridine, cooled to -20°C under an argon atmosphere, was added dropwise 2.23 g (1.5 mL, 15 mmol), of methyl dichlorophosphate. The reaction mixture was stirred for 5 min at -20°C until a white precipitate formed, and then 3.33 g (3.1 mL, 30 mmol) of thiophenol was added, followed by 3.05 g (4.2 mL, 30 mmol) of triethylamine. The mixture was stirred at -20°C for 3 h, after which it was poured into 150 mL of ice-water and extracted with 2 × 150 mL of $CHCl_3$. The chloroform extracts were combined, dried over Na_2SO_4, and the solvent was removed by rotary evaporation to give a pale yellow oil. The oil was redissolved in 30 mL $CHCl_3$, and 5.64 g (6.5 mL, 15 mmol) of cyclohexylamine was added. The solvent was again removed by rotary evaporation, and 200 mL of Et_2O was added to the oily residue to precipitate a white solid, which was collected by filtration, washed with Et_2O, and air-dried briefly. The product was further dried *in vacuo* to yield 4.53 g (79%) of cyclohexylammonium *S,S*-diphenylphosphorodithioate as a white, microcrystalline solid, mp 179-180°C. ^1H NMR (D_2O): δ 7.45 (4H, m), 7.26 (6H, m), 2.98 (1H, m, CHN), 1.81 (2H, m), 1.62 (2H, m), 1.46 (1H, m), 1.16 (4H, m), 1.00 (1H, m) ppm. ^{31}P NMR (D_2O): δ 36.88 (s) ppm.

2'-*O*-(*tert*-Butyldimethylsilyl)uridine 3',5'-bis(*S,S*-diphenyl)phosphoro-dithioate (7).[122] To a solution of 0.319 g (0.837 mmol) of cyclohexylammonium *S,S*-diphenylphosphorodithioate in 5.5 mL of dry pyridine under an argon atmosphere was added 0.355 g (1.12 mmol) of 2,4-mesitylenedisulfonyl chloride. The solution was stirred at room temperature for 0.5 h, then 0.100 g (0.279 mmol) of **6** was added. The reaction mixture was stirred for 1 h at room temperature, after which the reaction was quenched with the addition of 5

mL of H_2O. The resulting mixture was extracted with 3 × 10 mL of CH_2Cl_2. The extracts were combined, washed with 2 × 10 mL of 0.25 *M* triethylammonium bicarbonate (TEAB) and 2 × 10 mL of H_2O, dried over Na_2SO_4, and concentrated to dryness by rotary evaporation. The last traces of pyridine were removed from the oily residue by rotary evaporation with 3 × 10 mL of toluene, and the oil was dried further *in vacuo*. Flash chromatography on silica gel (4.5 × 15 cm), using EtOAc:$PhCH_3$ (1:2), resulted in the purification of 0.124 g (50%) of **7** as an off-white foam. TLC (EtOAc:$PhCH_3$, 1:2): R_f=0.24. ^{1}H NMR ($CDCl_3$): δ 9.55 (1H, br s, NH), 7.62-7.06 (21H, m), 6.00 (1H, d, H1'), 5.60 (1H, d, H5), 4.85 (1H, m, H4'), 4.35 (1H, m, H5'), 4.19 (1H, m, H5'), 4.14 (1H, t, H2'), 4.05 (1H, m, H3'), 0.82 (9H, s, $SiC(CH_3)_3$), 0.08 (3H, s, $SiCH_3$), 0.01 (3H, s, $SiCH_3$) ppm. ^{31}P NMR ($CDCl_3$): δ 49.26 (1P, s), 48.30 (1P, s) ppm.

2'-*O*-(*tert*-Butyldimethylsilyl)uridine 3',5'-bisphosphate (8).[123] To a solution of 16.5 mg (0.0188 mmol) of **7** in 1.5 mL of pyridine:water (2:1) was added 100 mg (0.599 mmol) of recrystallized silver acetate.[124] The reaction mixture was stirred at room temperature for 18 h, and then it was cooled to 0°C and treated with H_2S for 15 min, until a black precipitate had formed and a clear supernatant was produced. The excess H_2S was removed by rotary evaporation at ambient temperature, and the mixture was filtered through Celite to remove the precipitate, which was washed thoroughly with pyridine:water (2:1). The filtrate was reduced to dryness *in vacuo* to an off-white powder, which was dissolved in 0.05 *M* NH_4HCO_3, pH 7.9, and purified by anion-exchange chromatography on DEAE Sephadex A-25-120 (2 × 15 cm), using a 250-mL step gradient from 0.05 *M* to 0.5 *M* NH_4HCO_3, pH 7.9. The desired product eluted at ~0.4 *M* NH_4HCO_3, and lyophilization of the buffer, desalting with MeOH, drying *in vacuo*, and a second lyophilization from 0.1 *M* NH_4HCO_3, pH 7.9, afforded 9.1 mg (83%) of **8** as the ammonium salt, a white powder. ^{1}H NMR (D_2O): δ 7.86 (1H, d, H6), 5.88 (1H, d, H1'), 5.81 (1H, d, H5),

4.45 (1H, m, H4'), 4.36 (1H, m, H3'), 4.28 (1H, t, H2'), 3.97 (2H, m, H5'), 0.72 (9H, s, $SiC(CH_3)_3$), 0.02 (6H, s, $SiCH_3$) ppm.

Uridine 3',5'-bisphosphate (2).[125] To 32.0 mg (0.0546 mmol) of **8** was added 1.00 mL of a solution of tetrabutylammonium fluoride, 1.0 *M* in THF, with continuous stirring. The reaction mixture was stirred at room temperature for 0.5 h, and the solvent was removed by rotary evaporation. The residue was dried *in vacuo* and then applied to a DEAE Sephadex A-25-120 column (2 × 15 cm) in 2 mL of 0.05 *M* NH_4HCO_3, pH 8.0. A step gradient, total volume 350 mL, was eluted from 0.05 *M* to 0.7 *M* NH_4HCO_3, pH 8.0, and the product was obtained at approximately 0.45 *M* NH_4HCO_3. The buffer was removed by lyophilization, and the residue was desalted by coevaporation with MeOH, dried *in vacuo*, and lyophilized again from H_2O to yield 23.0 mg (89%) of **2** as the ammonium salt, a white powder. ^1H NMR (D_2O): δ 7.66 (1H, d, H6), 5.64 (1H, d, H1'), 5.60 (1H, d, H5), 4.19 (1H, m, H4'), 4.07 (1H, t, H2'), 4.03 (1H, m, H3'), 3.60 (2H, m, H5') ppm. ^{31}P NMR (D_2O): δ 3.62 (1P, d, P3'), 3.56 (1P, m, P5') ppm.

5-Chloromercuri-2'-*O*-(*tert*-butyldimethylsilyl)uridine 3',5'-bisphosphate (9).[6] To a solution of 100.0 mg (0.170 mmol) of **8** in 16 mL of 0.1 *M* sodium acetate, pH 6.0, was added 275 mg (0.864 mmol) of mercuric acetate. The reaction mixture was stirred at 60°C for 4 h. After cooling to room temperature, the mixture was cooled to 0°C, and a solution of 72.4 mg (1.70 mmol) of lithium chloride in 1 mL of H_2O was added. The reaction mixture was stirred at room temperature for 1 h and then extracted with 6 × 25 mL of EtOAc. The aqueous phase was diluted with 75 mL of cold EtOH, and the resulting suspension was allowed to stand at -20°C overnight. The product was isolated by centrifugation and washed with 2 × 30 mL of cold EtOH and 1 × 30

mL of cold Et_2O. The pellet was dried *in vacuo* and lyophilized from H_2O to give 98.3 mg (74%) of **9** as a dense white solid.

5-(3-Aminoallyl)-2'-*O*-(*tert*-butyldimethylsilyl)uridine 3',5'-bisphosphate (10).[6] To a solution of 53.8 mg (0.0692 mmol) of **9** in 4 mL of 0.1 *M* sodium acetate, pH 5.0, was added 0.41 mL of a solution containing 0.75 mL of allylamine (freshly distilled under argon) and 4.25 mL of glacial acetic acid, mixed at 0°C (0.082 mmol of allylamine was added). To this solution was added 23.2 mg (0.0712 mmol) of potassium tetrachloropalladate(II), and the reaction mixture was stirred at room temperature for 22 h, during which a black precipitate formed. The mixture was filtered through Celite, and the filter cake was washed with 2 × 5 mL of H_2O. The filtrate was applied to a DEAE Sephadex A-25-120 column (2 × 15 cm) equilibrated with 0.05 *M* NH_4HCO_3, pH 8.0. A 500 mL salt gradient was eluted from 0.05 *M* to 1.0 *M* NH_4HCO_3. The desired product eluted at 0.4-0.45 *M* NH_4HCO_3, and by-product uridine 3',5'-bisphosphate eluted at 0.5-0.55 *M* NH_4HCO_3. The buffer was removed from the fractions containing the desired product by lyophilization, and the residue was desalted with MeOH, dried *in vacuo*, and relyophilized from H_2O to give 21.7 mg (49%) of **10** as the ammonium salt, an off-white foam. ^1H NMR (D_2O): δ 8.08 (1H, s, H6), 6.44 (1H, d, Ur-CH=C-C-N), 6.36 (1H, m, Ur-C=CH-C-N), 5.87 (1H, d, H1'), 4.48 (m, H4'), 4.34 (t, H2'), 4.25 (m, H3'), 3.95 (2H, m, H5'), 3.56 (2H, d, Ur-C=C-CH_2-N), 0.75 (9H, s, $SiC(CH_3)_3$), -0.01,-0.03 (6H, 2s, $SiCH_3$) ppm.

3-Nitrophenoxyacetic acid.[127] A solution of 3.00 g (21.6 mmol) of 3-nitrophenol, 2.04 g (21.6 mmol) of chloroacetic acid, and 1.73 g (43.2 mmol) of sodium hydroxide in a minimum volume of H_2O (about 25 mL) was heated under reflux for 3 h. The reaction mixture was allowed

to cool to room temperature and was then acidified to pH 2.0 with concentrated HCl. The resulting mixture was extracted with 2 × 50 mL of Et_2O; the ether extracts were combined and re-extracted with 50 mL of 0.5 *M* Na_2CO_3. The Na_2CO_3 extract was then adjusted to pH 1.9 with concentrated HCl to precipitate an off-white solid, which was collected by filtration, washed with H_2O, and air-dried. Recrystallization from H_2O yielded 2.61 g (61%) of 3-nitrophenoxyacetic acid as pale yellow needles, mp 153-154°C. TLC (EtOH): R_f=0.52. ^{1}H NMR (DMSO-d_6): δ 7.87 (1H, dd, Ar-H4), 7.75 (1H, t, Ar-H2), 7.63 (1H, t, Ar-H5), 7.45 (1H, dd, Ar-H6), 4.92 (2H, s, CH_2) ppm. UV-VIS (10 m*M* Tris-HCl, pH 7.2): 226 (sh, ε=7100), 272 (ε=4200), 327 (ε=1400) nm.

5-(3-(3-Nitrophenoxy)acetamidoallyl)-2'-*O*-(*tert*-butyldimethylsilyl)uridine 3',5'-bisphosphate (11).[126] To a solution of 80.0 mg (0.406 mmol) of 3-nitrophenoxyacetic acid and 50.6 mg (0.439 mmol) of *N*-hydroxysuccinimide in 3.5 mL of CH_3CN under an argon atmosphere was added 90.7 mg (0.439 mmol) of 1,3-dicyclohexylcarbodiimide, and the reaction mixture was stirred at room temperature for 17 h. The mixture was filtered to remove dicyclohexylurea, and the filtrated was concentrated to dryness. The resulting *N*-hydroxysuccinimide ester of 3-nitrophenoxyacetic acid was dried *in vacuo* to a pale yellow foam and then dissolved in 1.0 mL of dry DMF. The DMF solution was added to a solution of 21.7 mg (0.0338 mmol) of **10** in 6.2 mL of 0.1 *M* $Na_2B_4O_7$, pH 8.5. The reaction mixture was stirred at room temperature for 3.5 h, during which a fine white precipitate formed. The suspension was applied directly to a DEAE Sephadex A-25-120 column (1 × 15 cm) equilibrated with 0.05 *M* NH_4HCO_3, pH 8.0, and a step gradient, volume 200 mL, was eluted from 0.05 *M* to 1.0 *M* NH_4HCO_3. Unreacted 3-nitrophenoxyacetic acid eluted at 0.3-0.4 *M* NH_4HCO_3, while the desired product eluted at 0.5-0.6 *M* NH_4HCO_3. The buffer was removed from the product by

lyophilization, and the product was desalted with MeOH, dried *in vacuo*, and lyophilized again from H_2O to afford 15.7 mg (57%) of **11** as the ammonium salt, an off-white powder. ^1H NMR (D_2O): δ 7.76 (2H, m, H6 and Ar-H4), 7.63 (1H, m, Ar-H2), 7.39 (1H, t, Ar-H5), 7.23 (1H, m, Ar-H6), 6.26 (1H, dt, Ur-C=CH), 6.06 (1H, d, Ur-CH=C), 5.85 (1H, d, H1'), 4.52 (2s, $ArOCH_2$), 4.43 (m, H4'), 4.35 (m, H3'), 4.30 (m, H2'), 3.99 (2H, m, H5'), 3.82 (2H, br d, Ur-C=C-CH_2), 0.62 (9H, s, $SiC(CH_3)_3$), -0.05 (6H, s, $SiCH_3$) ppm.

5-(3-(3-Nitrophenoxy)acetamidoallyl)uridine 3',5'-bisphosphate (12).[125] To 13 mg (0.016 mmol) of **11** was added 1.0 mL of a solution of tetrabutylammonium fluoride, 1.0 *M* in THF, with continuous stirring. The reaction mixture was stirred at room temperature for 0.5 h, and then the solvent was removed by rotary evaporation. The residue was dried *in vacuo* for 1 h, dissolved in 2 mL of H_2O, and extracted with 3 × 2 mL of CH_2Cl_2. The aqueous phase was then applied to a DEAE Sephadex A-25-120 column (1 × 15 cm), and the product was purified by a 200 mL step gradient from 0.05 *M* to 1.0 *M* NH_4HCO_3, pH 8.0. A single UV-absorbing band eluted at 0.45-0.55 *M* NH_4HCO_3, and the buffer was removed from these fractions by lyophilization. The residue was desalted by rotary evaporation with MeOH four times, dried *in vacuo*, and lyophilized from H_2O to yield 10.2 mg (90%) of **12** as the ammonium salt, a pale yellow powder. ^1H NMR (D_2O): δ 7.79 (1H, s, H6), 7.76 (1H, m, Ar-H4), 7.65 (1H, m, Ar-H2), 7.42 (1H, t, Ar-H5), 7.26 (1H, m, Ar-H6), 6.28 (1H, dt, Ur-C=CH), 6.13 (1H, d, Ur-CH=C), 5.85 (1H, d, H1'), 4.56 (2s, $ArOCH_2$), 4.47 (m, H4'), 4.30 (t, H2'), 4.25 (m, H3'), 3.95 (2H, m, H5'), 3.84 (2H, br d, Ur-C=C-CH_2) ppm.

5-Chloromercuriuridine 5'-triphosphate (13).[6] To a solution of 100 mg (0.171 mmol) of uridine 5'-triphosphate in 18 mL of 0.1 *M* NaOAc, pH 6.0, was added 276 mg (0.866

mmol) of mercuric acetate. The reaction mixture was heated at 60°C with continuous stirring for 4 h, after which it was first cooled to room temperature and then to 0°C. A solution of 72.3 mg (1.71 mmol) of lithium chloride in 1 mL of H_2O was added, and the reaction mixture was allowed to stir for 1 h while warming to room temperature. The reaction mixture was then extracted with 6 × 30 mL of EtOAc, and the product was precipitated from the aqueous phase with the addition of 60 mL of ice cold EtOH. The resulting suspension was allowed to stand at -20°C overnight, and the product was collected by centrifugation at 4°C, washed with 2 × 25 mL of cold EtOH and 1 × 25 mL of cold Et_2O. The dense white solid was dried *in vacuo* and dissolved in 5 mL of H_2O, causing the solution to turn black, and lyophilized to give 133.6 mg (>100%) of **13** as the lithium salt, a light gray powder. The black color of the solution of **13** and the light gray color of the final isolated product are probably caused by a trace amount of reduced mercury in the compound. This small amount of mercury is not deleterious to the next reaction in the sequence, so the product may be used without further work.

5-(3-Aminoallyl)uridine 5'-triphosphate (1).[6] To a solution of 133.6 mg of **13** in 10 mL of 0.1 *M* NaOAc, pH 5.0, was added 1.07 mL (2.13 mmol) of a 2 *M* solution of allylamine in 4 *M* AcOH. Then 58.7 mg (0.180 mmol) of potassium tetrachloropalladate(II) was added, and the reaction mixture was stirred at room temperature for 22 h. The reaction mixture was filtered through a 0.45-μ nitrocellulose membrane, after which the filtrate was allowed to stand at 4°C overnight and was refiltered through a 0.2-μ nitrocellulose membrane. The filtrate was applied to a DEAE Sephadex A-25-120 column (2 × 15 cm) prewashed with 0.1 *M* NaOAc, pH 5.0. A 350-mL step gradient was eluted from 0.1 M to 0.7 *M* TEAB, pH 7.6, and the desired product was collected at approximately 0.5 *M* TEAB. Lyophilization of the buffer, desalting by coevaporation with MeOH, and a second lyophilization from H_2O afforded 31.8 mg (20% based on uridine

5'-triphosphate) of **1** as the triethylammonium salt, an off-white solid. ^{1}H NMR (D_2O): δ 8.01 (1H, s, H6), 6.45 (1H, d, Ur-CH=C-C-N), 6.33 (1H, dt, Ur-C=CH-C-N), 5.87 (1H, d, H1'), 4.33-4.20 (3H, m, H2', H3', H4'), 4.16 (2H, m, H5'), 3.59 (2H, d, Ur-C=C-CH_2-N), 3.05 (24H, q, $NH(CH_2C)_3^+$), 1.17 (36H, t, $NH(CCH_3)_3^+$) ppm. ^{31}P NMR (D_2O): δ -10.26 (1P, d, P_γ), -11.25 (1P, d, P_α), -22.71 (1P, t, P_β) ppm.

HPLC Purification of 5-(3-Aminoallyl)uridine 5'-triphosphate (1).[137] The purity of **1**, following DEAE Sephadex chromatography, was assayed by strong anion-exchange HPLC and determined to be 89.4%. Among the impurities were starting material (**13**) and uridine 5'-triphosphate, formed by reduction of the starting material, which accounted for a total of 3.8% of the composition of the product. An aliquot of 1 mg of **1** was then purified by strong anion-exchange HPLC, using the same conditions as the analytical assay. Strong anion-exchange HPLC was performed at ambient temperature on a SynChrom SynChropak Q-300 column, 4.1 × 250 mm, using a linear gradient from 0-0.4 *M* $(NH_4)_2CO_3$, pH 9.0, in 30 min. The column was preequilibrated with H_2O and the gradient was started 10 min after injection. Detection of the constituents was accomplished by monitoring the absorbance of the eluent at both 260 nm (λ_{max} for **13**) and 290 nm (λ_{max} for **1**). At a flow rate of 1.0 mL/min, compound **1** elutes at approximately 40.8 min under these conditions. After HPLC purification, the purity of **1** was again assayed and determined to be 95.8%, with the only impurities being the monophosphate (2.0%) and diphosphate (2.2%) derivatives of 5-(3-aminoallyl)uridine, produced by hydrolysis of the triphosphate group during purification and isolation at pH 9.0.

HPLC separation of 2'-*O*-(*tert*-butyldimethylsilyl)uridine 3',5'-bisphosphate (8) and uridine 5'-monophosphate, 3'-cyclic phosphate.[137] Strong anion-exchange

HPLC separation of the constituents of the product mixture obtained during attempted synthesis of **8** was performed at ambient temperature using a SynChrom SynChropak Q-300 column, 4.1 × 250 mm. The column was preequilibrated with H_2O, and 10 min after injection, a linear gradient from 0-0.6 *M* $(NH_4)_2CO_3$, pH 9.0, was eluted in 30 min. Detection was accomplished by monitoring the absorbance of the eluent at 270 nm, λ_{max} for uridine. At a flow rate of 1.0 mL/min, compound **8** elutes with a retention time of approximately 27.7 min, while uridine 5'-monophosphate, 3'-cyclic phosphate, a potential by-product of the synthesis of **8**, elutes at about 25.8 min. The resolution of these two compounds under these conditions is sufficient to allow for preparative purification of **8**, but at pH 9.0 the silica-based anion-exchange resin in the column is slowly degraded until the column is rendered unusable. Therefore, this volatile buffer system is not practical for all preparative HPLC separations with the Q-300 column, but some applications of limited scale and duration are feasible (see above).

Transformation of Riboprobe Gemini™ Positive Control Template plasmid DNA.[138] Template DNA for *in vitro* transcription reactions was transformed into *Escherichia coli* strain HB101 in a manner similar to that employed for plasmid pBR322. An overnight culture of *E. coli* strain HB101 in 5 mL of L-Broth was inoculated into 100 mL of L-Broth and incubated with shaking until the $OD_{590} \cong 0.2$ (2 h 40 min). The culture was then chilled in an ice-water bath for 5 min, and the cells were collected by centrifugation at 8000 rpm for 5 min, 4°C. The pellets were resuspended in a total volume of 40 mL of 0.1 *M* $CaCl_2$ and allowed to stand on ice for 20 min. After a second centrifugation at 8000 rpm, 4°C, for 5 min, the pellets were resuspended in a final total volume of 1 mL of 0.1 *M* $CaCl_2$ and kept on ice for 24 h. To a suspension of 100 μL of these cells was added 5 μL of a solution of Riboprobe Gemini™ Positive Control Template plasmid DNA (Promega Biotec), 100 μg/mL in H_2O, and 9 μL of TCM (10 m*M* Tris-HCl, pH 8.0, 10 m*M*

$CaCl_2$, 10 m*M* $MgCl_2$). This mixture was kept on ice for 15 min, heat shocked at 37°C for 5 min, and allowed to stand at room temperature for 5 min. Then 2 mL of L-Broth was added, and the culture was incubated at 30°C with shaking for 2 h. A 100 μL aliquot of the transformed culture was then removed and streaked onto L-plates, ampicillin- (Ap, 50 μg/mL) and tetracycline-substituted (Tc, 15 μg/mL) agar plates in dilutions of 1:10, 1:100, and 1:1000. After overnight incubation at 37°C, cells of the correct phenotype (Ap resistant, Tc sensitive) grew well, and single colonies were picked from the 1:1000 dilution Ap-plate, restreaked onto fresh plates, and incubated again at 37°C overnight. This selective pressure process was repeated once more, and then a single colony of *E. coli* containing pGEM™ Positive Control Template DNA was inoculated into 5 mL of Ap-substituted (50 μg/mL) L-Broth and grown overnight at 37°C with shaking. This liquid culture was repeated several times with a 1:100 dilution of the old culture every 12 h, and to each of two 1 mL aliquots of these cells was added dimethylsulfoxide (DMSO) to 7%. These two liquid cultures were then stored at -80°C.

Preparation of Riboprobe Gemini™ Positive Control Template plasmid DNA.[139] Milligram quantities of pGEM™ Positive Control Template DNA were grown in *E. coli* strain HB101 and isolated by procedures similar to those employed for plasmid pBR322. An aliquot of 4 mL of a 5-mL overnight culture of *E. coli* strain HB101 in Ap-substituted (50 μg/mL) L-Broth was inoculated into 1 L of Vogel-Bonner minimal media (2 g/L Casamino acids, 20 mL/L 50X Vogel-Bonner salts, 50 mg/L thymine, 4 g/L *D*-glucose, 50 mg/L ampicillin, 1 mg/L thiamine-HCl). This culture was incubated at 37°C with shaking until the OD_{590}≅0.7-0.9 (OD_{590}=0.833 after 6.5 h). The culture was then amplified with 2 mL of chloramphenicol solution (Chloromycetin sodium succinate, Parke-Davis, 100 mg/mL) and incubated at 37°C with shaking for 17 h.

The culture was then chilled in an ice-water bath with frequent agitation, after which the cells were harvested by centrifugation at 5000 rpm, 4°C, for 20 min. The pellet was resuspended in 4-6 mL of a solution of 25% sucrose in 50 m*M* Tris-HCl, pH 8.0, and transferred to a Ti70 polycarbonate centrifuge tube. Then 1.6 mL of lysozyme solution (5 mg/mL, prepared fresh) was added. The culture was allowed to stand on ice for 5 min with occasional swirling, and 5 mL of 0.5 *M* EDTA, pH 8.0, was added. The culture was again kept on ice for 5 min with occasional swirling. A solution of 10% Triton X-100, 50 m*M* Tris-HCl, pH 8.0, and 60 m*M* EDTA, pH 8.0, was added to fill the centrifuge tube almost completely, and the culture was shaken vigorously until the lysate formed a clear supernatant. The lysed cells were collected by centrifugation at 30,000 rpm, 4°C, for 30 min. The supernatant was transferred to a 50-mL polypropylene centrifuge tube, and 350 µL of DNase-free[144] Ribonuclease A solution (2 mg/mL) was added. This mixture was allowed to incubate at 37°C for 20 min, after which about one-third volume (~6-7 mL) of a solution containing 30% PEG-8000 in 1.5 *M* NaCl was added. The lysate was mixed by shaking, incubated on ice for 8 h, and centrifuged at 6000 rpm, 4°C, for 20 min. The pellet was resuspended in 2-5 mL of cold 10X TE (100 m*M* Tris-HCl, 10 m*M* EDTA, pH 8.0) and combined with a mixture of 59.52 g CsCl, 3.2 mL of ethidium bromide (EtBr) solution (10 mg/mL), and 1X TE to give a final weight of 124.00 g. (This equals 80 mL of 48% w/w CsCl and has $\rho \cong 1.55$.) The mixture was stirred to dissolve the CsCl, loaded into a VTi50 quick-seal centrifuge tube, and the first CsCl banding run was performed at 42,000 rpm, 17°C, for 20 h.

The lower (plasmid) band was removed with a syringe, using UV illumination to detect the EtBr-stained DNA. The plasmid DNA was transferred to a VTi65 quick-seal centrifuge tube, the tube was filled with the CsCl banding solution described above ($\rho \cong 1.55$, except containing 400 µg/mL of EtBr), and the second CsCl banding run was performed at 55,000 rpm, 17°C, for 12 h. Again, the plasmid band was removed with a syringe, using UV illumination. The EtBr was

extracted from the aqueous phase with 15 equal volumes of isoamyl alcohol saturated with 1X TE. The isolated plasmid DNA was then dialyzed against 1X STE (100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA) for 56 h, with buffer changes at 4, 8, 12, 24, 48, 52, and 54 h, to give 451 μg of pGEM™ Positive Control Template DNA, which was stored at -20°C.

Cleavage of template DNA with restriction endonucleases.[140] To a typical 25 μL EcoRI reaction mixture, containing 2 μg of pGEM™ Positive Control Template DNA, 22 μL of H_2O, and 2.5 μL of 10X EcoRI buffer (1 *M* Tris-HCl, pH 7.5, 500 m*M* NaCl, 50 m*M* $MgCl_2$), or to a typical 25 μL Hinc II reaction mixture, containing 2 μg of pGEM™ DNA, 18 μL of H_2O, 2.5 μL of 10 m*M* DTT, and 2.5 μL of 10X Hinc II buffer (100 m*M* Tris-HCl, pH 7.4, 1 *M* NaCl, 70 m*M* $MgCl_2$), was added 8 units of the appropriate enzyme. The reaction mixture was incubated at 37°C for 2 h, after which the extent of cleavage was assayed by agarose minigel electrophoresis. Upon completion of the digestion, the reaction mixture was diluted to 50 μL with H_2O and extracted with one volume each of phenol, phenol:chloroform (1:1), and chloroform. The linearized template was then isolated by EtOH precipitation, dessicated to dryness *in vacuo*, and stored at -20°C.

***In vitro* transcription synthesis of RNA from linear DNA templates.**[63-65]

Transcription by T7 RNA polymerase II.[64,65] Linear template DNA was prepared by EcoRI digestion of Riboprobe Gemini™ Positive Control Template to give a termination site 43 base pairs downstream from the T7 promoter site. This linearized template was dissolved in H_2O at a concentration of ~0.1 μg/μL, and 5 μL of this stock solution was used in a typical 50 μL (VOL_{pre}) transcription reaction. To 5 μL of the template stock solution were added sequentially: 5

μL each of stock solutions of ATP, GTP, and UTP (5 m*M* in 70 m*M* Tris-HCl, pH 7.5), 0.5 μL of a stock solution of CTP (5 m*M* in 70 m*M* Tris-HCl, pH 7.5), 5 μL of 100 m*M* DTT, 1.5 μL of RNasin (40 u/μL), 16 μL of H_2O, 1 μL of 5'-[α-^{32}P]CTP solution (>410 Ci/mmol, 10 mCi/mL), 5 μL of 10X transcription buffer, and 1 μL of T7 RNA polymerase II (85-125 u/μL). The 10X buffer which consistently gave the highest yields of RNA contained 400 m*M* Tris-HCl, pH 7.5, 200 m*M* NaCl, 120 m*M* $MgCl_2$, and 20 m*M* spermidine-3HCl. When aminoallyl-RNA was synthesized, 2.5 μL of a stock solution of 5-(3-aminoallyl)uridine 5'-triphosphate (**1**), 10 m*M* in 10 m*M* Tris-HCl, pH 7.5, was used in place of UTP, and the amount of H_2O added was adjusted accordingly.

The radioactivity in a 1 μL aliquot of the reaction mixture (CPM_{pre}) was measured by Cerenkov counting, and the remainder of the mixture was incubated at 37°C for 2 h. Then 1 μL of a solution of RNase-free deoxyribonuclease I in 0.15 *M* NaCl (1 u/μL) was added, and the reaction mixture was incubated at 37°C for 10 min. The mixture was then extracted with an equal volume of phenol:chloroform (1:1). The aqueous layer was diluted to 125 μL with H_2O and applied to a Sephadex G-50-80 column (1 mL) equilibrated with TE, pH 7.4. The labeled transcript was eluted from the Sephadex by centrifugation at approximately 2000 rpm, 25°C, for 2 min. The elution volume (VOL_{post}) of the RNA solution was measured, and the radioactivity of a 1 μL aliquot of this eluent (CPM_{post}) was measured by Cerenkov counting. The yield of transcript was then calculated using the following equation:

$$\text{Yield } (\mu g \text{ RNA}) = 4(\mu g \text{ CTP}_{pre})(VOL_{post})(CPM_{post})/(VOL_{pre})(CPM_{pre}). \quad (1)$$

The RNA solution was diluted with H_2O, as necessary, to a concentration of ≤1.5 mR/h per μL and stored at -20°C. The transcript products from this reaction were analyzed by high-resolution

denaturing gel electrophoresis on 15% polyacrylamide, 1:20 crosslinked, sequence determination gels, 0.4 × 400 mm, containing 50% urea. Electrophoresis was carried out at 1600 V for 6.5 h to achieve nucleotide resolution of transcripts in the size vicinity of the anticipated 43-nucleotide product. Autoradiography was carried out at -80°C with the use of an intensification screen.

Transcription by SP6 RNA polymerase II.[63] Linear template DNA was prepared by Hinc II digestion of Riboprobe Gemini™ Positive Control Template to give a termination site 172 base pairs downstream from the SP6 promoter site. This linearized template was dissolved in H_2O at a concentration of ~0.1 μg/μL, and 5 μL of this stock solution was used in a typical 50 μL (VOL_{pre}) transcription reaction. To 5 μL of the template stock solution were added sequentially: 5 μL each of stock solutions of ATP, GTP, and UTP (5 m*M* in 70 m*M* Tris-HCl, pH 7.5), 0.5 μL of a stock solution of CTP (5 m*M* in 70 m*M* Tris-HCl, pH 7.5), 5 μL of 100 m*M* DTT, 1.5 μL of RNasin (40 u/μL), 10 μL of H_2O, 1 μL of 5'-[α-^{32}P]CTP solution (>410 Ci/mmol, 10 mCi/mL), 10 μL of 5X transcription buffer or 5 μL of 10X transcription buffer, and 2 μL of SP6 RNA polymerase II (10 u/μL). Several transcription buffers, described on p. 29, were tried in this assay, but there was no buffer system which consistently gave measurable yields of RNA as calculated from equation (1). Two of the buffer systems (buffers A and B on p. 29) were sucessfully employed on two separate occasions in the synthesis of as much as 220 ng of the 172-nucleotide transcript per μg of template, but this yield could only be reproduced with the freshest lots of SP6 RNA polymerase II.

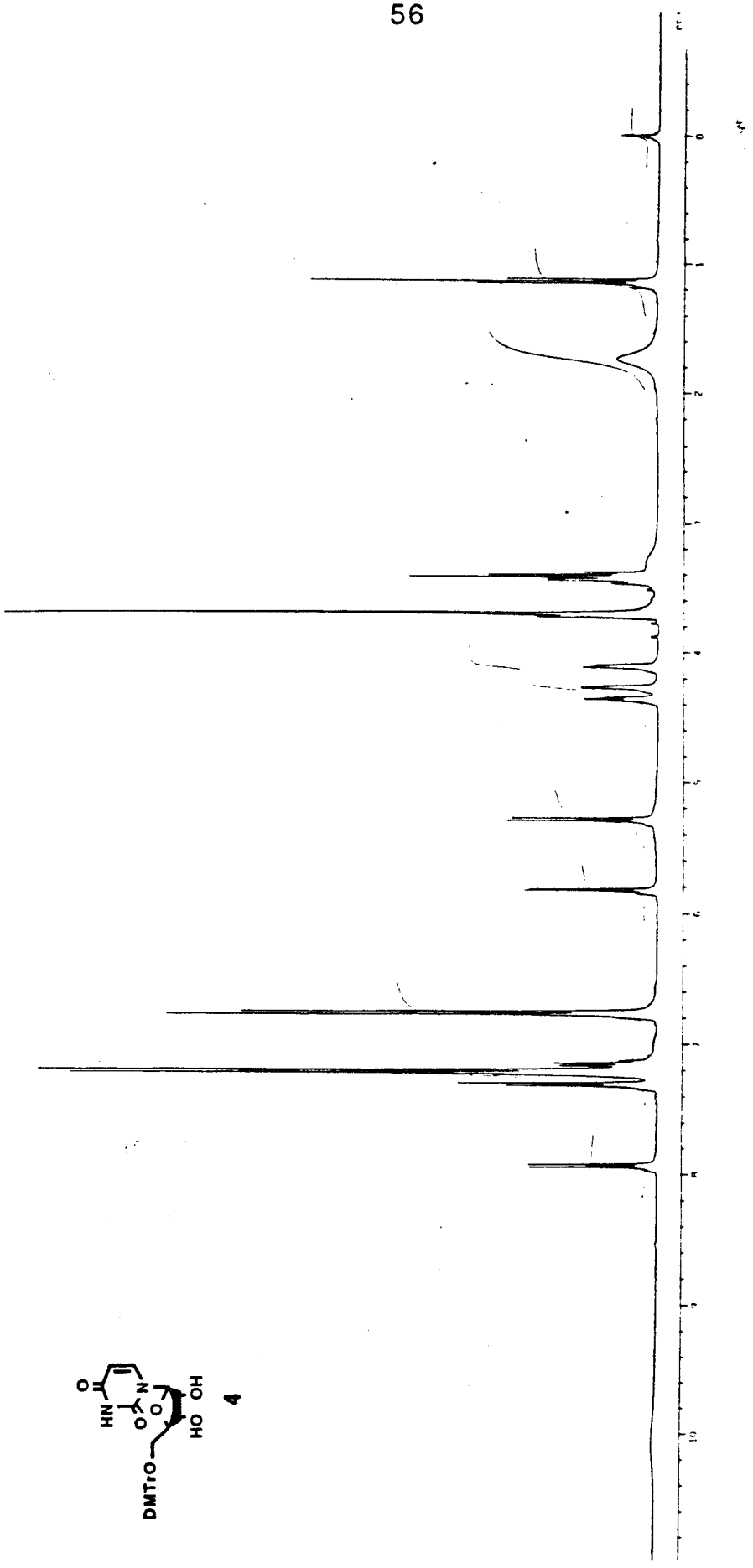
Modification of template DNA end structure.[141,142] Riboprobe Gemini™ Positive Control Template DNA was linearized by digestion with EcoRI restriction endonuclease, and the linear template product was dissolved in H_2O at a concentration of ~0.1 μg/μL.

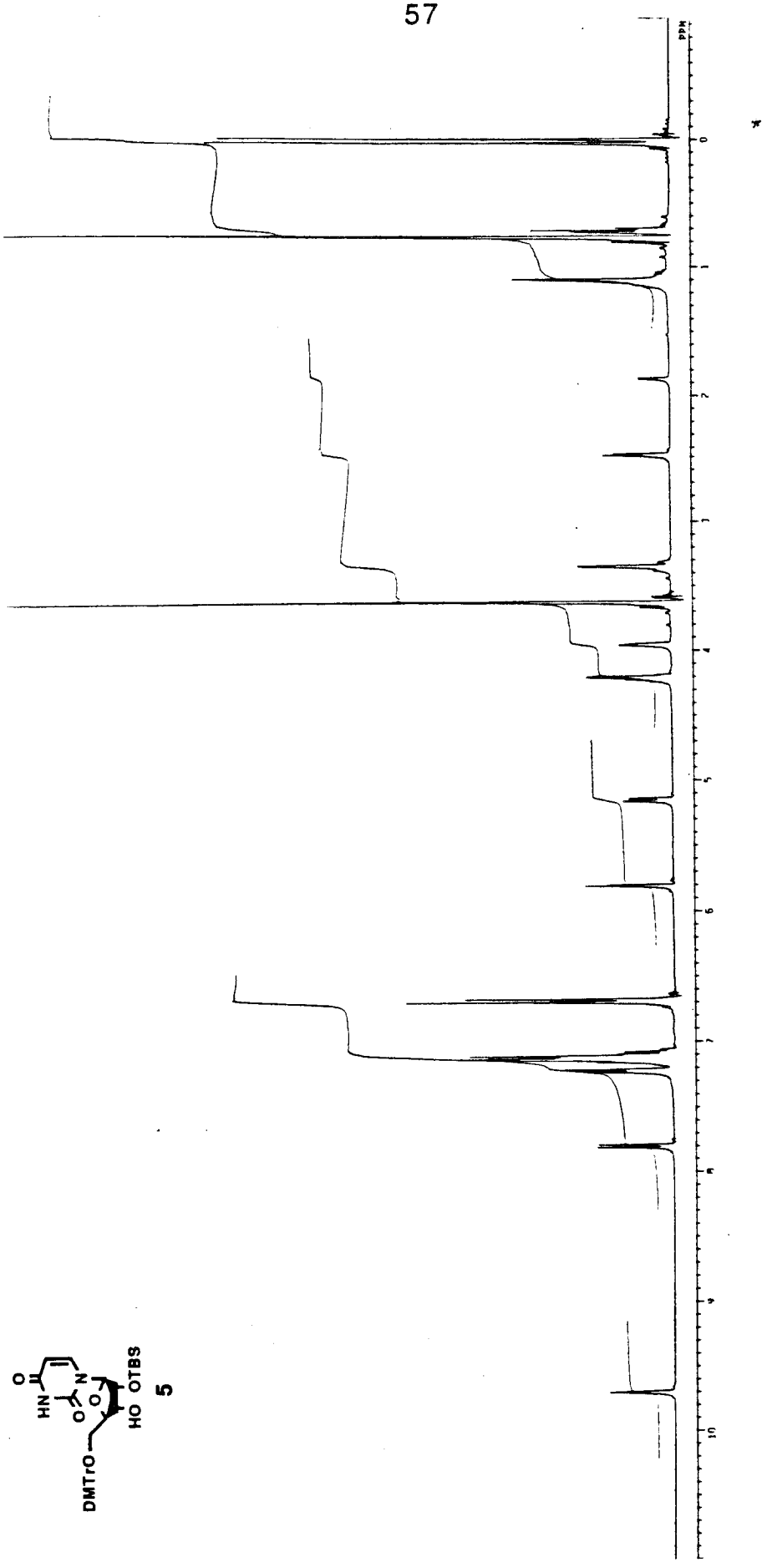
Klenow fragment of DNA polymerase I.[141] To an aliquot of 5 μL of the stock solution of EcoRI-linearized template DNA (~0.1 μg/μL) were added the following reagents sequentially: 65 μL of H_2O, 10 μL of 50 m*M* DTT, 2 μL each of stock solutions of dATP, dGTP, dTTP, and dCTP (10 m*M* in H_2O), 10 μL of 10X Hae III buffer (60 m*M* Tris-HCl, pH 7.6, 500 m*M* NaCl, 60 m*M* $MgCl_2$), and 2 μL of Klenow enzyme (5 u/μL). The reaction mixture was incubated for 30 min at 37°C, after which an additional 1 μL of Klenow enzyme was added, and the mixture was incubated again for 30 min at 37°C. The reaction mixture was extracted with one volume each of phenol, phenol:chloroform (1:1), and chloroform. The modified template DNA was isolated by EtOH precipitation, dessicated to dryness *in vacuo*, checked for purity by agarose minigel electrophoresis, and stored at -20°C.

Nuclease S1.[142] To a 5 μL aliquot of the stock solution of EcoRI-linearized template DNA (~0.1 μg/μL) were added 29 μL of H_2O, 10 μL of TA (500 m*M* Tris-HCl, 500 m*M* NaOAc), pH 4.7, 5 μL of 5 m*M* $ZnSO_4$, and 1 μL of Nuclease S1 (1 u/μl). The reaction mixture was incubated at 37°C for 5 min, after which it was extracted with equal volumes of phenol, phenol:chloroform (1:1), and chloroform. The modified linear template was isolated by EtOH precipitation, dessicated to dryness *in vacuo*, assayed by agarose minigel electrophoresis, and stored at -20°C.

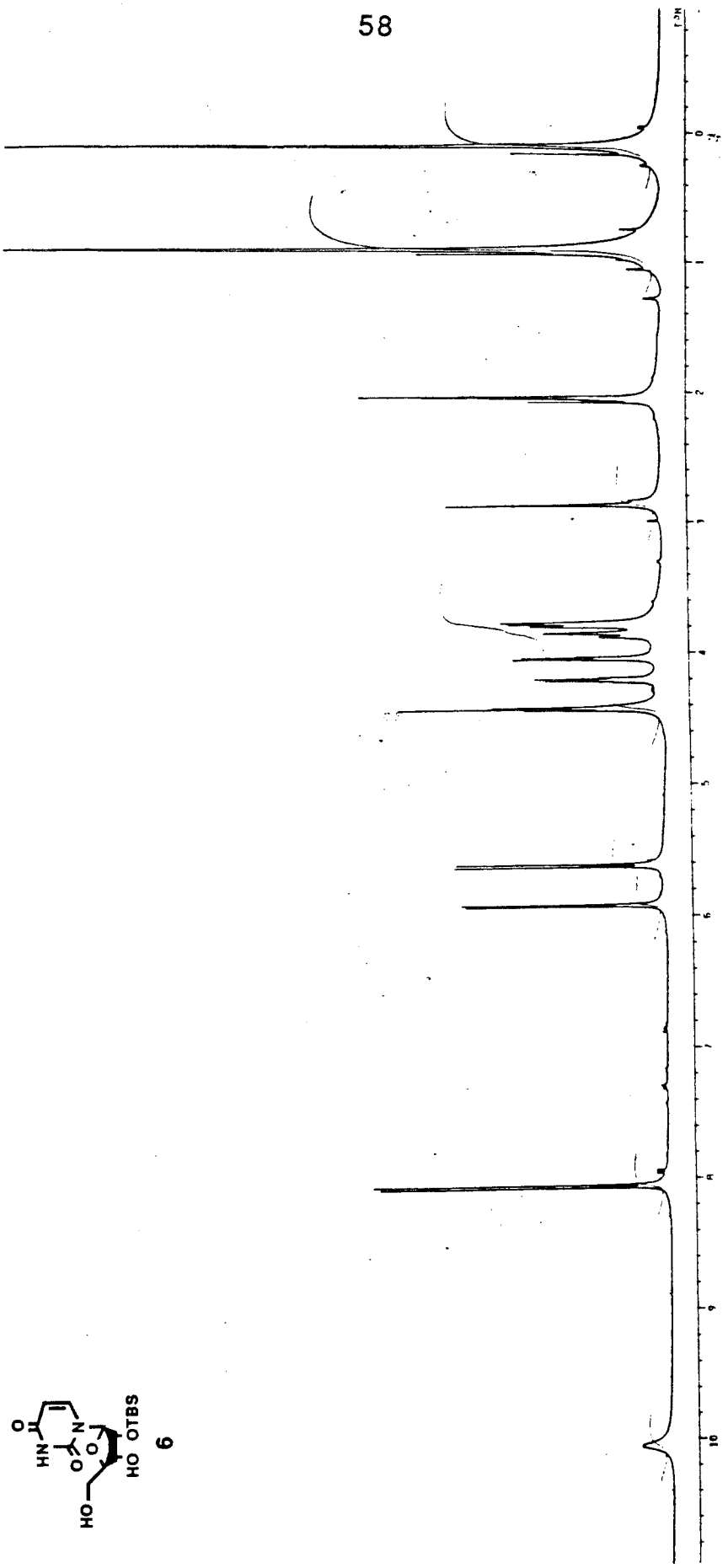
NMR

SPECTRA

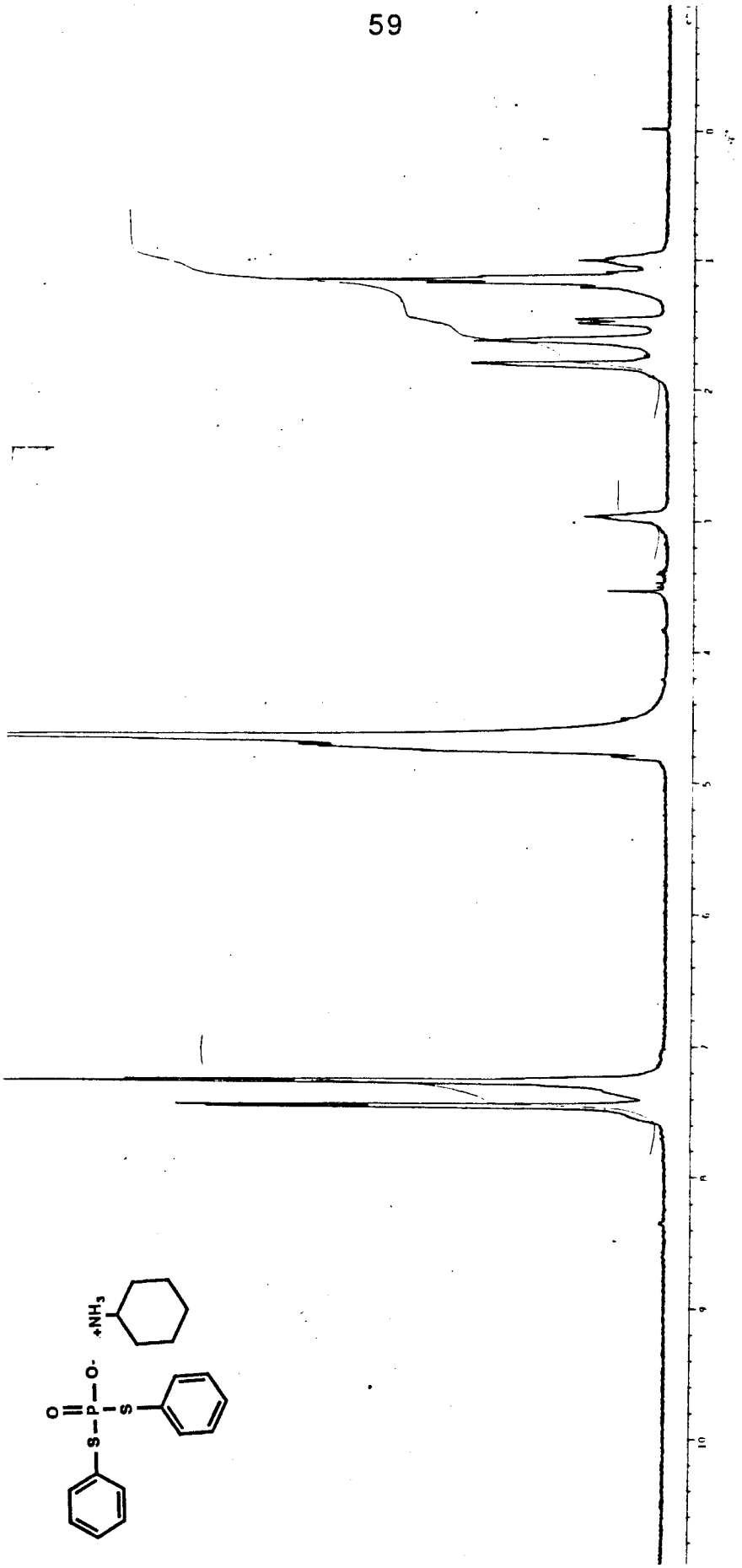

DMTrO
HO OH
HN
O
O
N
O
4

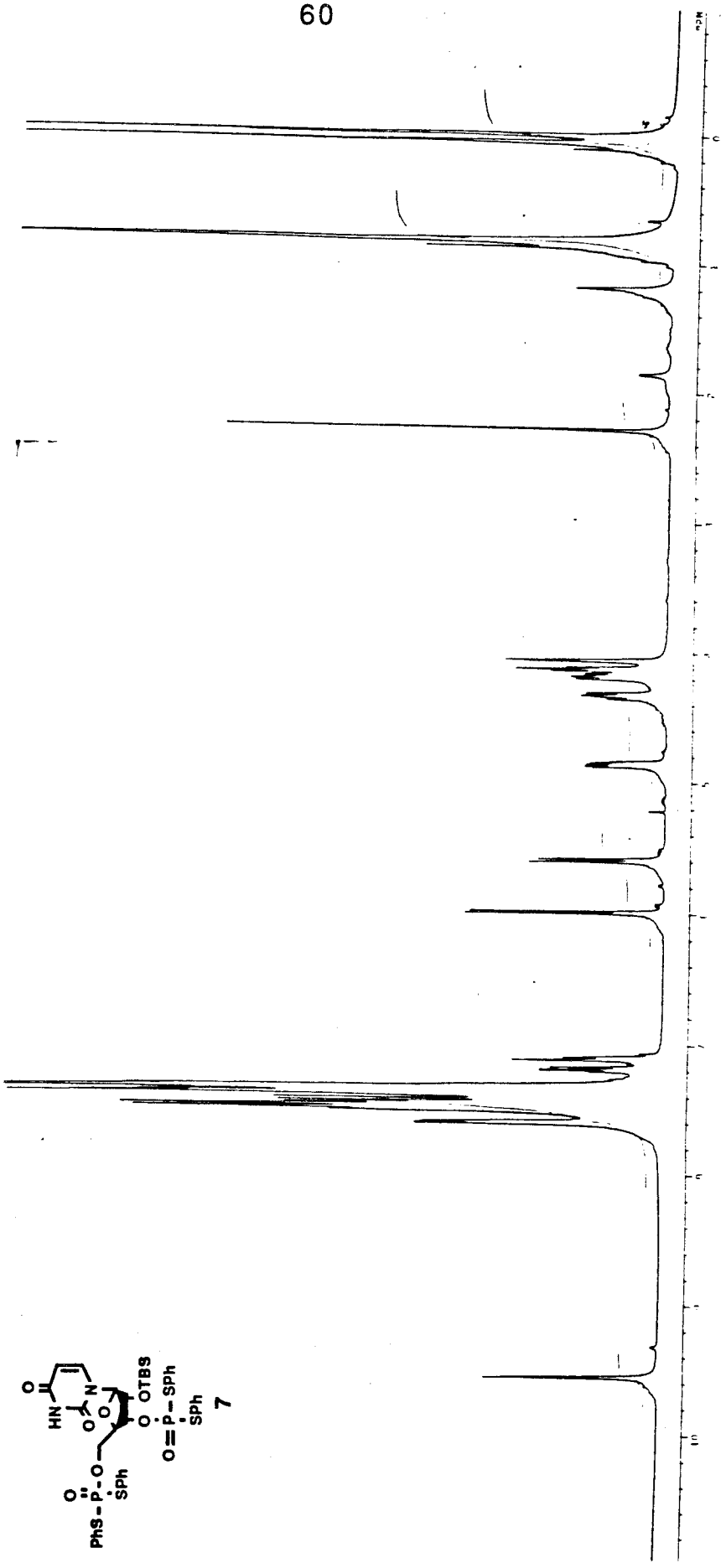


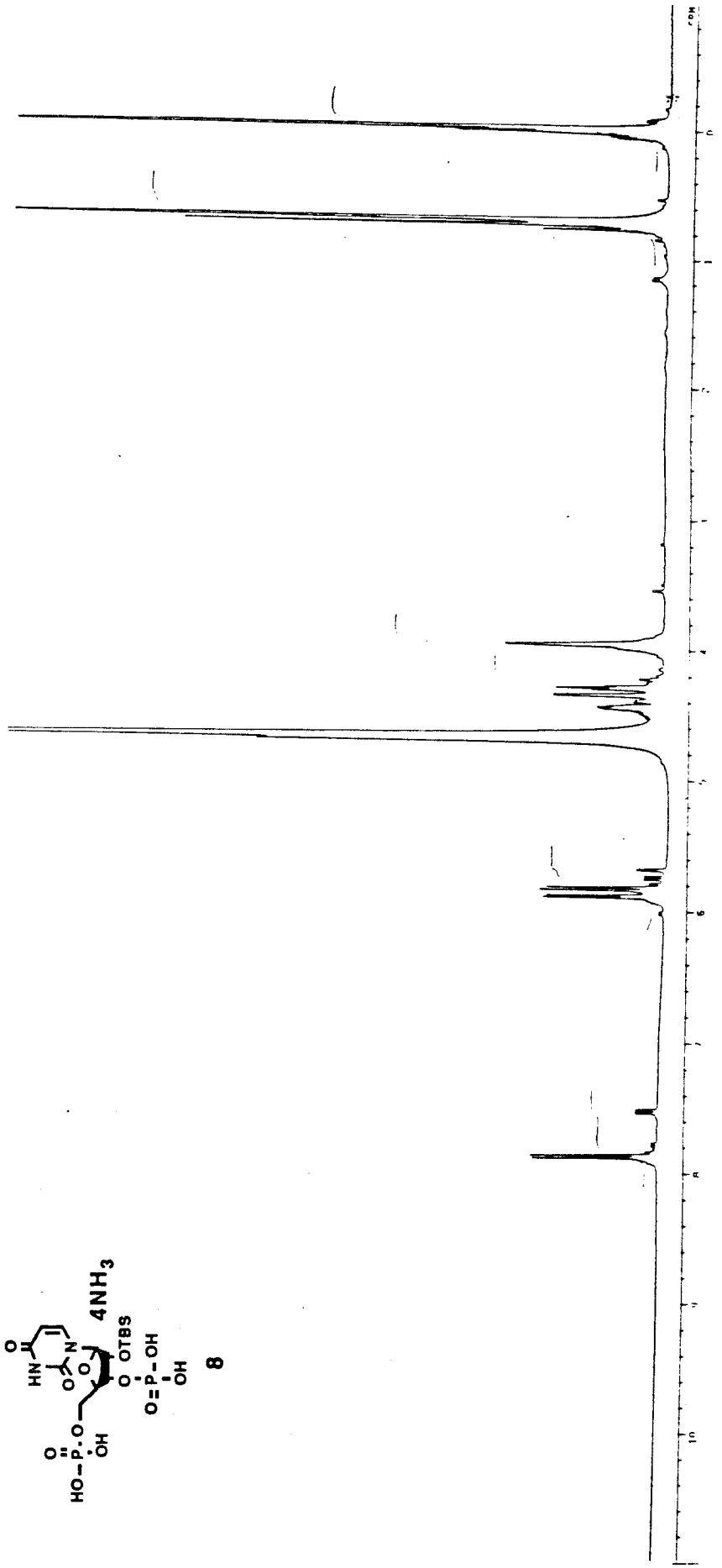
5

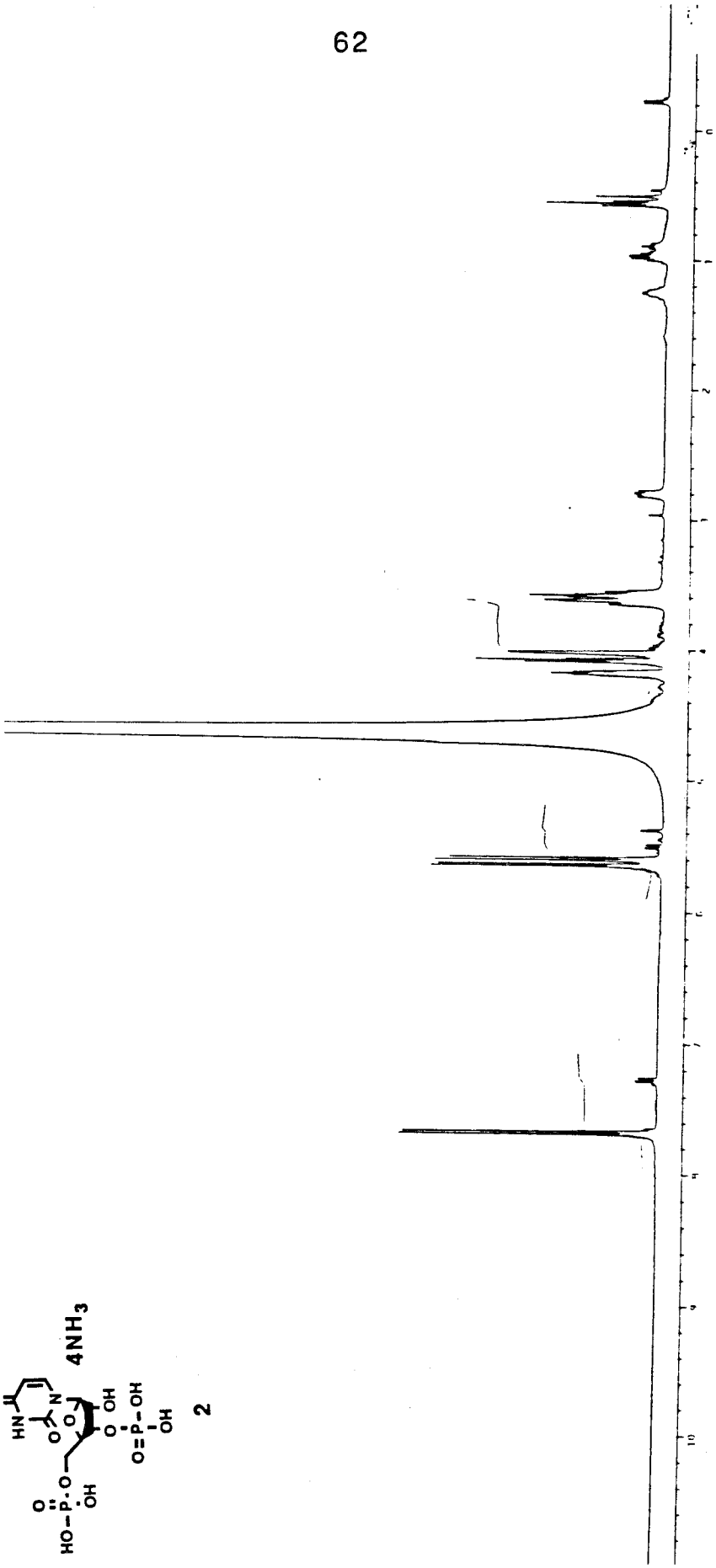


6

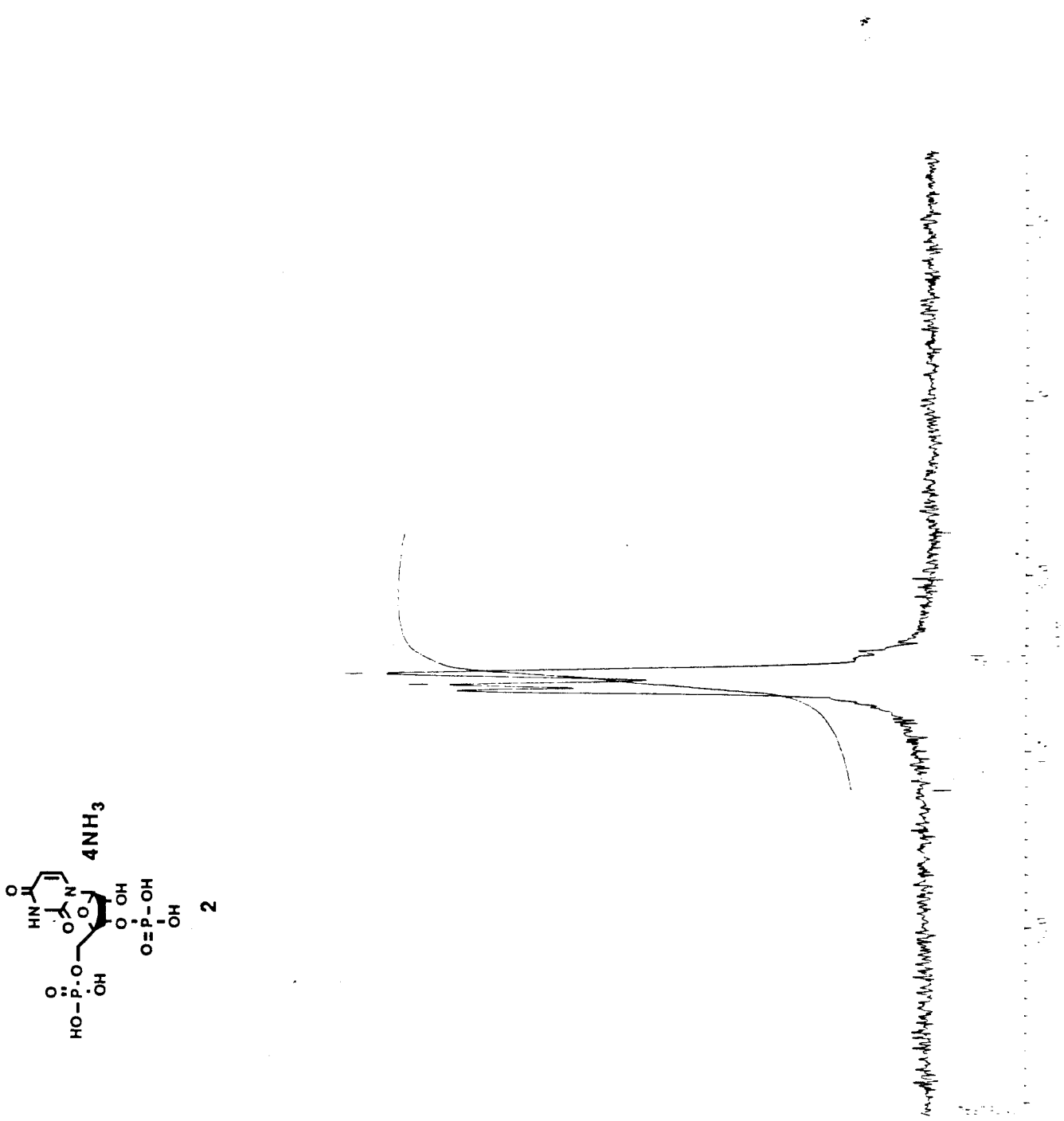

+NH3
O
O-
P
S
S

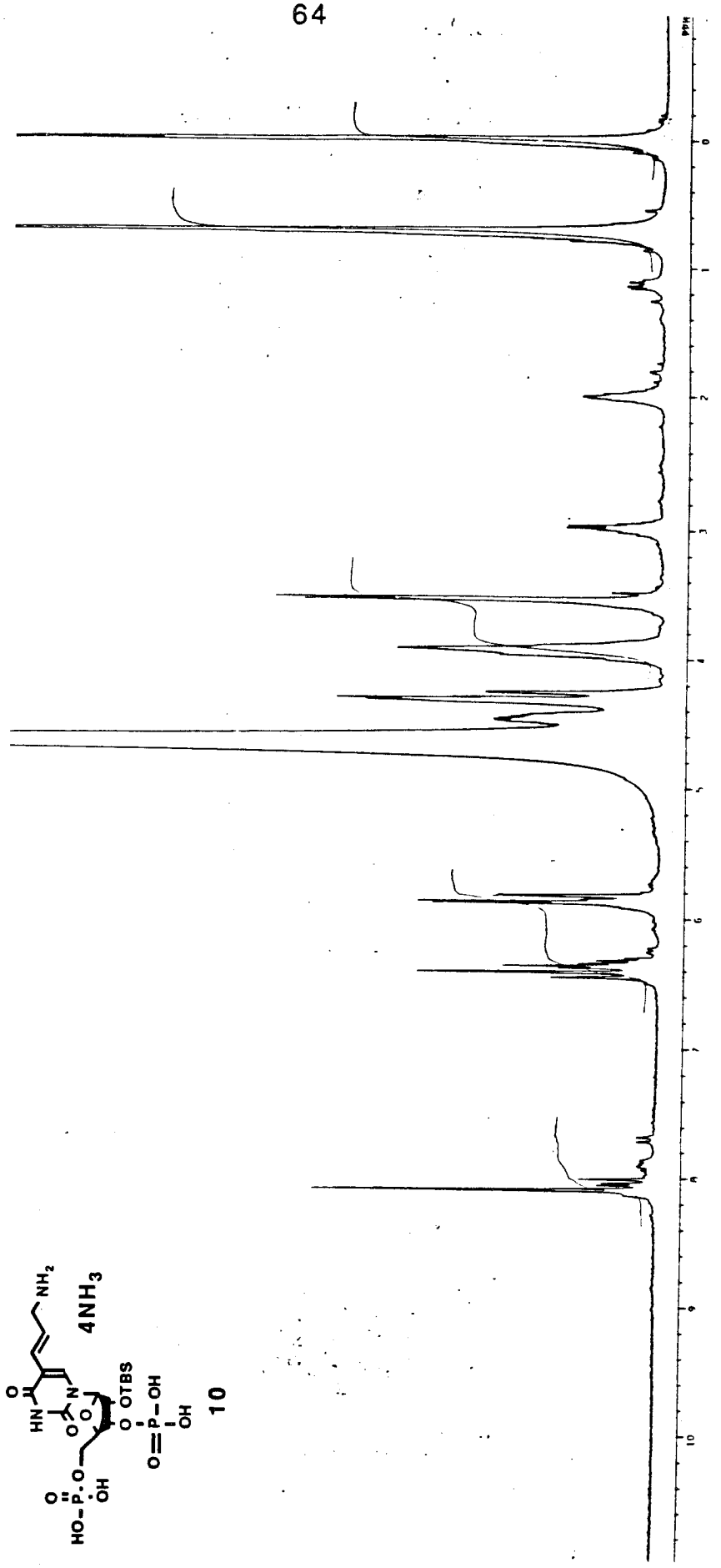

O
HN
N
O
O
O
OTBS
O=P-SPh
SPh
PhS-P-O
SPh
7

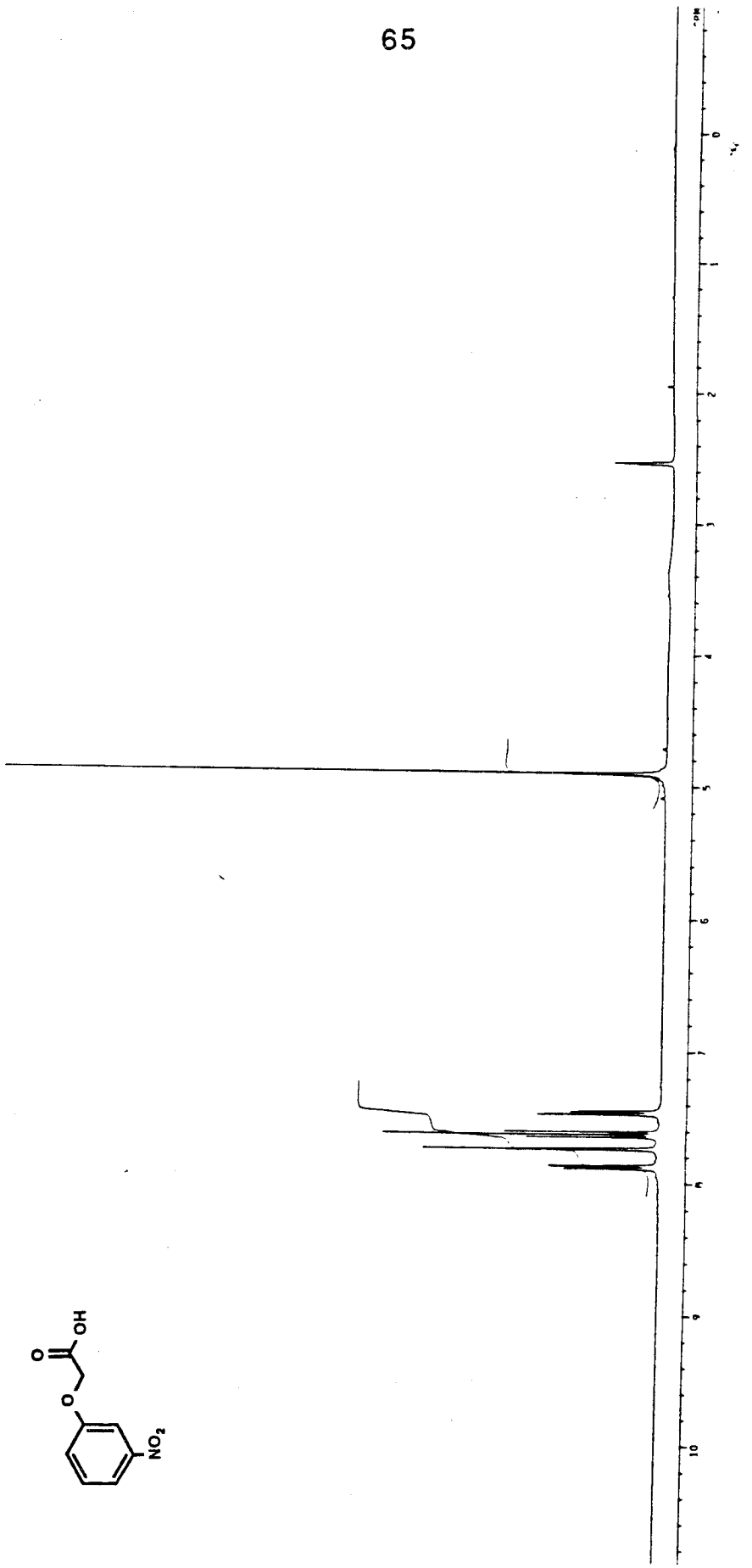

4NH3
OTBS
8

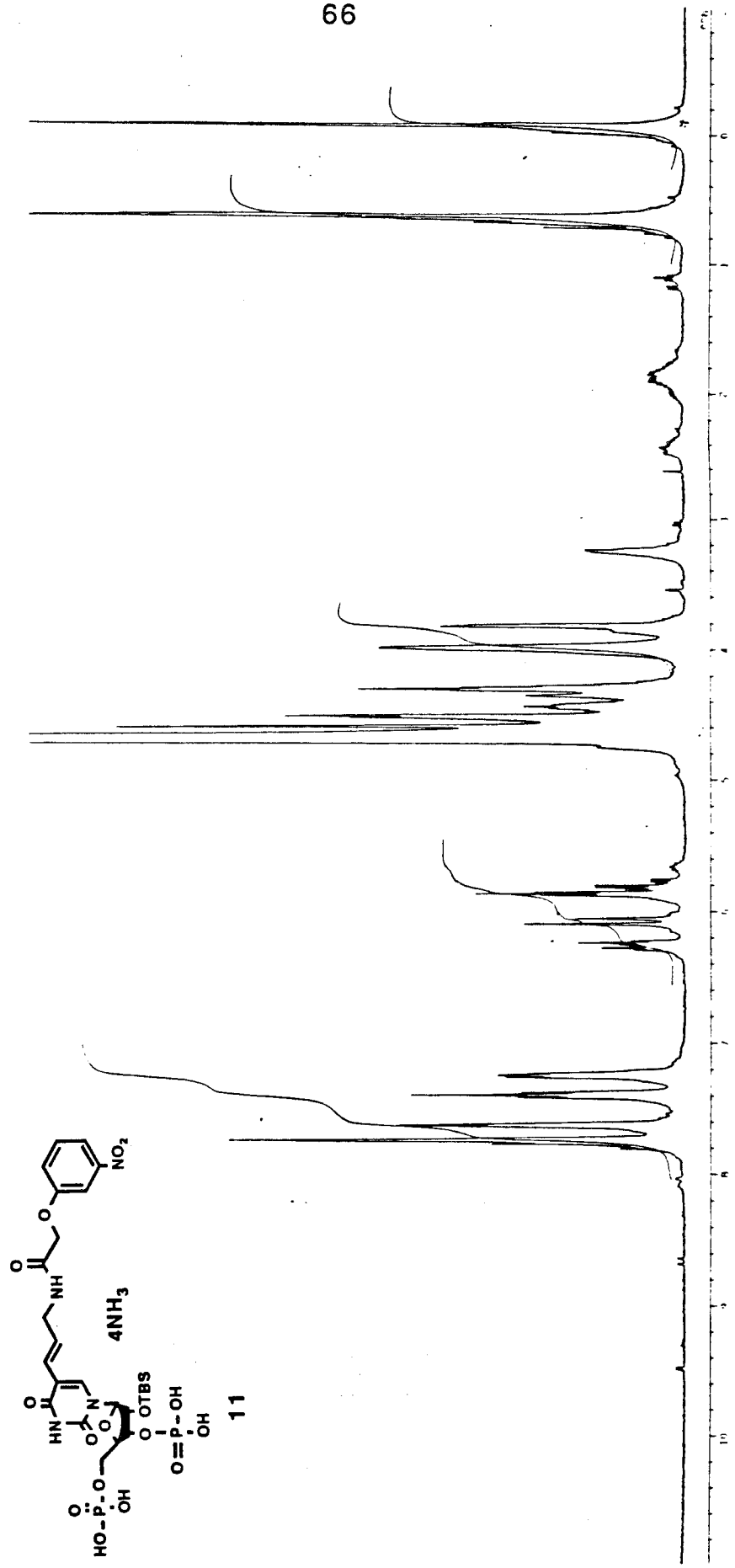


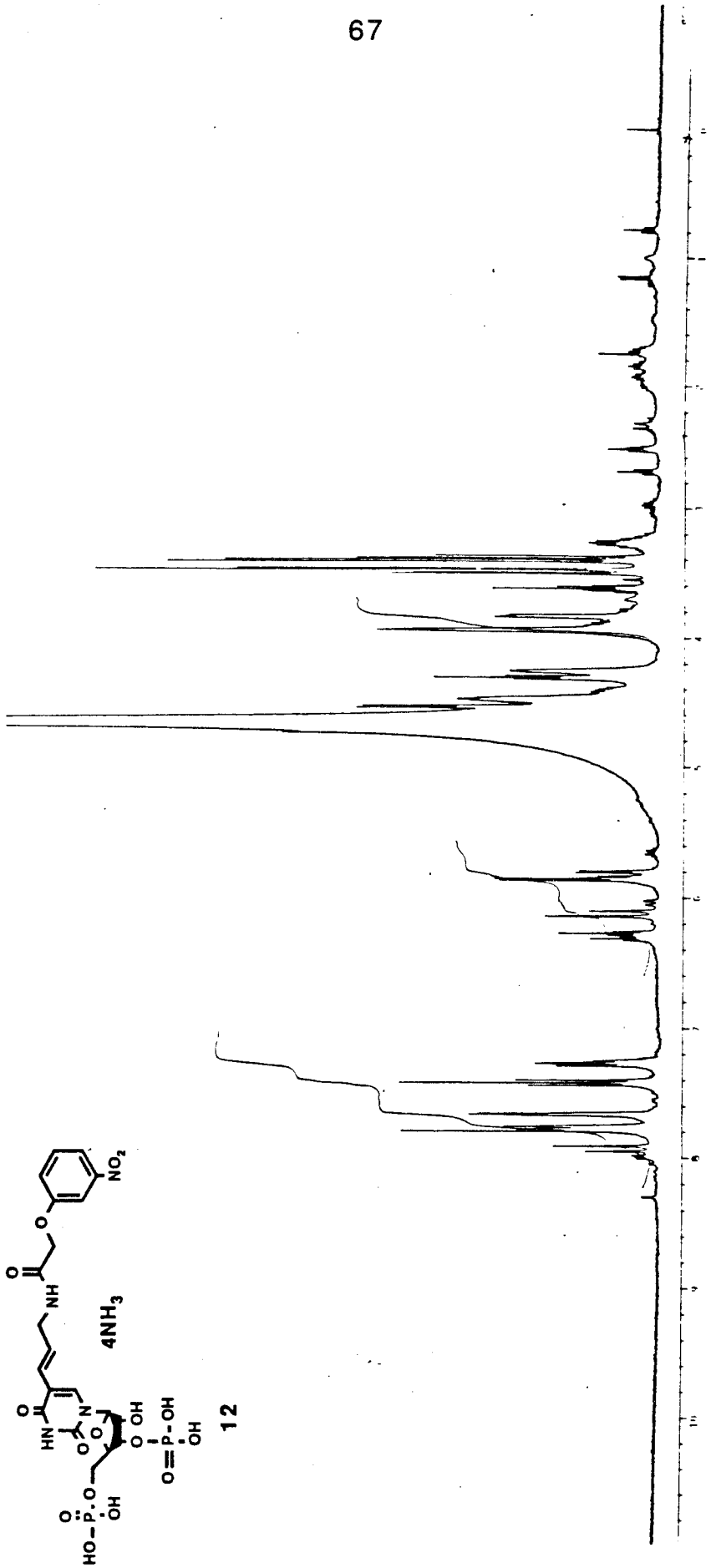
2 $4NH_3$

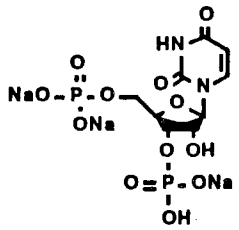

4NH3
2


4NH3
10

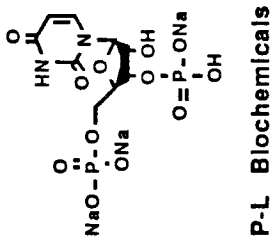

PPM
0
1
2
3
4
5
6
7
8
9
10
O
OH
O
NO2


4NH3
11

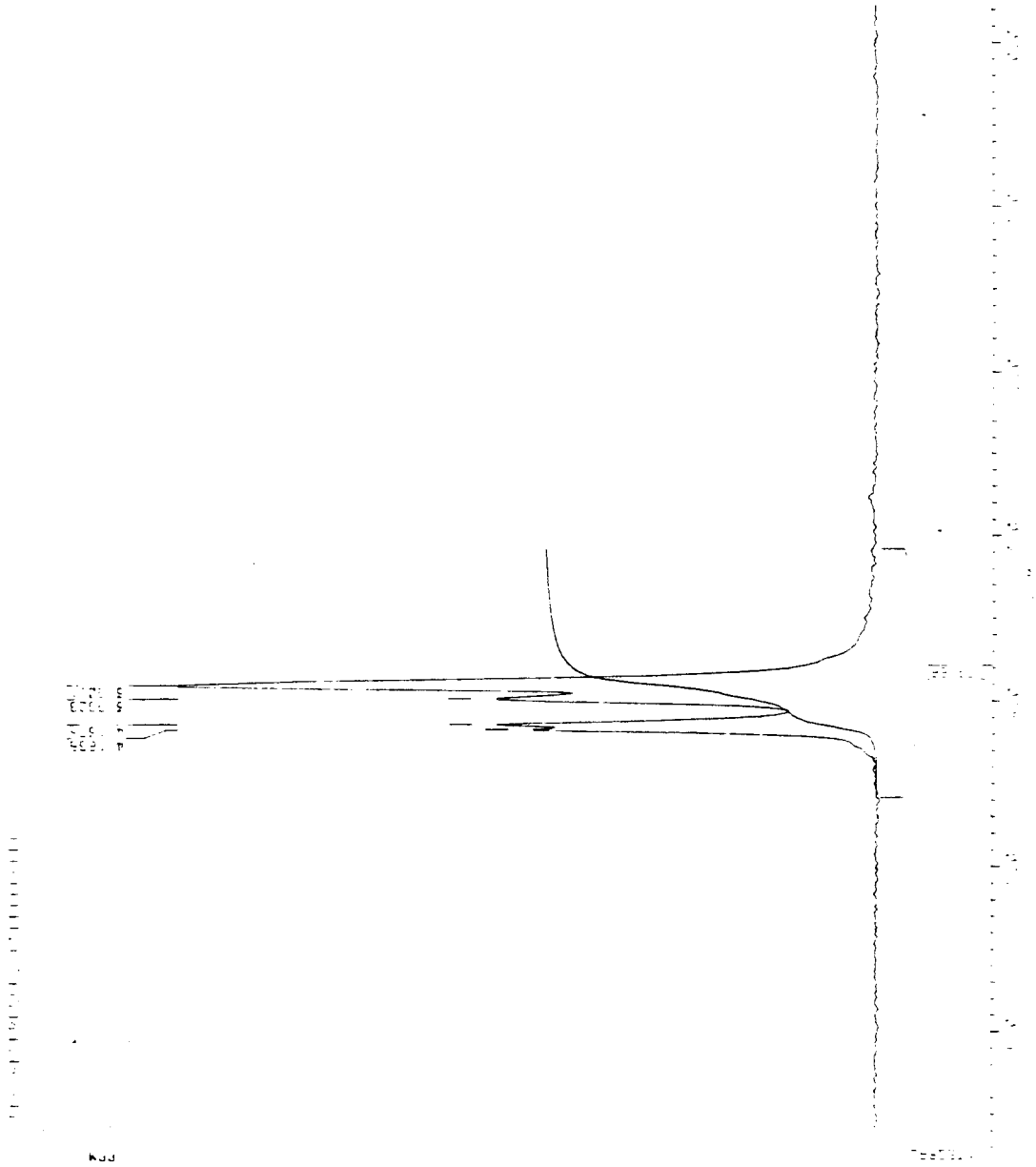

4NH3
12

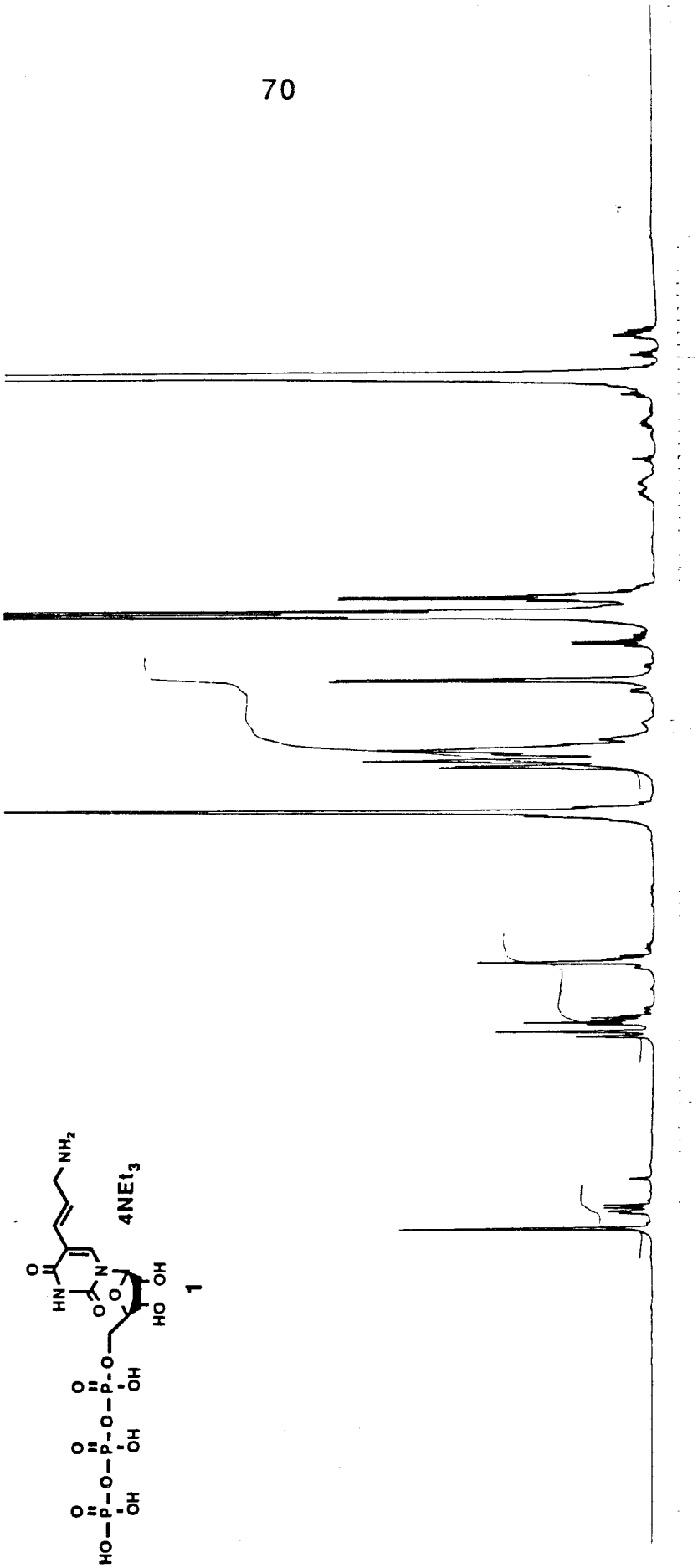


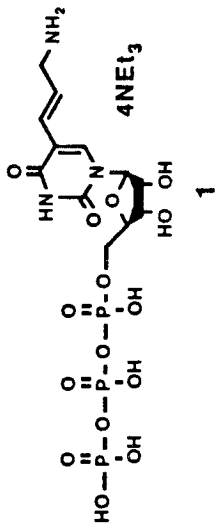
P-L Biochemicals

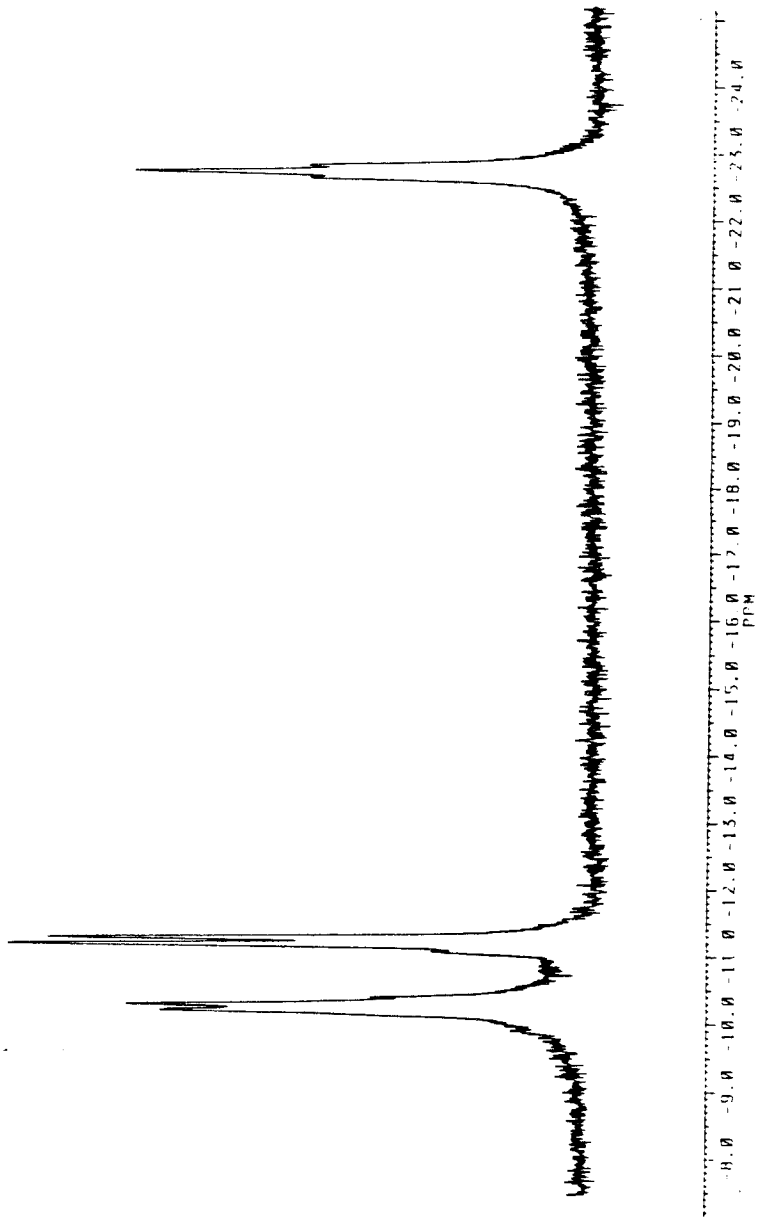


P-L Biochemicals




NH2
4NEt3
HN
O
N
HO OH
HO—P—O—P—O—P—O
OH OH OH
1


NH2
4NEt3
HO OH
1


-8.0 -9.0 -10.0 -11.0 -12.0 -13.0 -14.0 -15.0 -16.0 -17.0 -18.0 -19.0 -20.0 -21.0 -22.0 -23.0 -24.0
PPM

References

1. Asseline, U., Thoung, N.T., Helene, C., *C. R. Acad. Sci. (Paris)*, **1983**, *297*, 369-372.
2. Asseline, U., Toulme, F., Thuong, N.T., Delarue, M., Montenay-Garestier, T., Helene, C., *EMBO J.*, **1984**, *3*, 795-800.
3. Asseline, U., Delarue, M., Lancelot, G., Toulme, F., Thuong, N.T., Montenay-Garestier, T., Helene, C., *Proc. Natl. Acad. Sci. USA*, **1984**, *81*, 3297-3301.
4. Lancelot, G., Asseline, U., Thuong, N.T., Helene, C., *Biochemistry*, **1985**, *24*, 2521-2529.
5. Helene, C., unpublished research.
6. Langer, P.R., Waldrop, A.A., Ward, D.C., *Proc. Natl. Acad. Sci. USA*, **1981**, *78*, 6633-6637.
7. Leary, J.J., Brigati, D.J., Ward, D.C., *ibid.*, **1983**, *80*, 4045-4049.
8. Minning, J.E., Hershey, N.D., Broker, T.R., Pellegrini, M., Mitchell, H.K., Davidson, N., *Chromosoma (Berlin)*, **1975**, *53*, 107-117.
9. Broker, T.R., Angerer, L.M., Yen, P.H., Hershey, N.D., Davidson, N., *Nucleic Acids Res.*, **1978**, *5*, 363-384.
10. Sodja, A., Davidson, N., *ibid.*, 385-401.
11. Eshaghpour, M., Soll, D., Crothers, D.M., *ibid.*, **1979**, *7*, 1485-1495.
12. Draper, D.E., Gold, L., *Biochemistry*, **1980**, *19*, 1774-1781.
13. Draper, D.E., *Nucleic Acids Res.*, **1984**, *12*, 989-1002.
14. Bauman, J.G.J., Wiegant, J., Van Duijn, P., *J. Histochem. Cytochem.*, **1981**, *29*, 227-237.
15. Bauman, J.G.J., Wiegant, J., Van Duijn, P., *ibid.*, 238-246.

16. Cosstick, R., McLaughlin, L.W., Eckstein, F., *Nucleic Acids Res.*, **1984**, *12*, 1791-1810.

17. Richardson, R.W., Gumport, R.I., *ibid.*, **1983**, *11*, 6167-6184.

18. Grineva, N.I., Karpova, G.G., *FEBS Lett.*, **1973**, *32*, 351-355.

19. Grineva, N.I., Karpova, G.G., Kuznetsova, L.M., Venkstern, T.V., Bayev, A.A., *Nucleic Acids Res.*, **1977**, *4*, 1609-1631.

20. Summerton, J., Bartlett, P.A., *J. Mol. Biol.*, **1978**, *122*, 145-162.

21. Salganik, R.I., Dianov, G.L., Ovchinnikova, L.P., Voronina, E.N., Kokoza, E.B., Mazin, A.V., *Proc. Natl. Acad. Sci. USA*, **1980**, *77*, 2796-2800.

22. Dianov, G.L., Mazin, A.V., Zaitsev, B.N., Vavilin, V.I., Salganik, R.I., *Mol. Biol.*, **1980**, *14*, 203-205.

23. Martin, R.F., Haseltine, W.A., *Science (Washington, D.C.)*, **1981**, *213*, 896-898.

24. Dreyer, G.B., Dervan, P.B., *Proc. Natl. Acad. Sci. USA*, **1985**, *82*, 968-972.

25. Chu, B.C.F., Orgel, L.E., *ibid.*, 963-967.

26. Hall, B.D., Spiegelman, S., *Proc. Natl. Acad. Sci., Wash.*, **1961**, *47*, 137-146.

27. Gillespie, D., Spiegelman, S., *J. Mol. Biol.*, **1965**, *12*, 829-842.

28. John, H., Birnstiel, M., Jones, K., *Nature (London)*, **1969**, *223*, 582-587.

29. Gall, J., Pardue, M., *Proc. Natl. Acad. Sci. USA*, **1969**, *63*, 378-383.

30. Milman, G., Langridge, R., Chamberlin, M.J., *ibid.*, **1967**, *57*, 1804-1810.

31. Wang, A.H.J., Fujii, S., Van Boom, J.H., Van der Morel, G.A., Van Boeckel, S.A.A., Rich, A., *Nature (London)*, **1982**, *299*, 601-604.

32. Zimmerman, S.B., Pheiffer, B.H., *Proc. Natl. Acad. Sci. USA*, **1981**,*78*, 78-82.

33. Arnott, S., Fuller, W., Hodgson, A., Prutton, I., *Nature (London)*, **1968**, *220*, 561-564.

34. Bonner, J., Kung, G., Bekhor, I., *Biochemistry*, **1967**, *6*, 3650-3653.

35. McConaughy, B.L., Laird, C.D., McCarthy, B.J., *ibid.*, **1969**, *8*, 3289-3295.

36. Birnstiel, M.L., Sells, B.H., Purdon, I.F., *J. Mol. Biol.*, **1972**, *63*, 21-39.

37. Schmeckpeper, B.J., Smith, K.D., *Biochemistry*, **1972**, *11*, 1319-1326.

38. Casey, J., Davidson, N., *Nucleic Acids Res.*, **1977**, *4*, 1539-1552.

39. Chien, Y.H., Davidson, N., *ibid.*, **1978**, *5*, 1627-1637.

40. Thomas, M., White, R.L., Davis, R.W., *Proc. Natl. Acad. Sci. USA*, **1976**, *73*, 2294-2298.

41. Vogelstein, B., Gillespie, D., *Biochem. Biophys. Res. Commun.*, **1977**, *75*, 1127-1132.

42. Lee, C.S., Davis, R.W., Davidson, N., *J. Mol. Biol.*, **1970**, *48*, 1-22.

43. White, R.L., Hogness, D., *Cell*, **1977**, *10*, 177-192.

44. Wei, C.F., Tsao, Y.K., Robberson, D.L., Gotto, Jr., A.M., Brown, K., Chan, L., *J. Biol. Chem.*, **1985**, *260*, 15211-15221.

45. Kaback, D.B., Angerer, L.M., Davidson, N., *Nucleic Acids Res.*, **1979**, *6*, 2499-2517.

46. Jones, F.S., Grimberg, J.I., Fischer, S.G., Ford, J.P., *Gene*, **1985**, *39*, 77-83.

47. Cech, T.R., Rio, D.C., *Proc. Natl. Acad. Sci. USA*, **1979**, *76*, 5051-5055.

48. Andersen, N., Thill, G.P., Kramer, R.A., *Mol. Cell Biol.*, **1983**, *3*, 562-569.

49. Chow, L.T., Roberts, J.M., Lewis, J.B., Broker, T.R., *Cell*, **1977**, *11*, 819-836.

50. Neuwald, P.D., Meyer, J., Maizel, J.V., Westphal, H., *J. Virol.*, **1977**, *21*, 1019-1030.

51. Meyer, J., Neuwald, P.D., Lai, S.P., Maizel, J.V., Westphal, H., *ibid.*, 1010-1018.

52. Kramer, R.A., Cameron, J.R., Davis, R.W., *Cell*, **1976**, *8*, 227-232.

53. Kaback, D.B., Rosbash, M., Davidson, N., *Proc. Natl. Acad. Sci. USA*, **1981**, *78*, 2820-2824.

54. Wittig, B., Wittig, S., *Biochem. Biophys. Res. Commun.*, **1979**, *91*, 554-562.

55. Born, J., Wittig, B., Wittig, S., *Mol. Biol. Rep.*, **1980**, *6*, 159-161.

56. Tyler, B.M., Adams, J.M., *Proc. Aust. Biochem. Soc.*, **1979**, *12*, 85.

57. *Idem.*, *Gene*, **1980**, *10*, 147-155.

58. Cooper, J.A., Moss, B., *Nucleic Acids Res.*, **1979**, *6*, 3599-3612.

59. Woolford, J.L., Rosbash, M., *ibid.*, 2483-2497.

60. Ogilvie, K.K., Nemer, M.J., *Tetrahedron Lett.*, **1980**, *21*, 4159-4162.

61. Ogilvie, K.K., Nemer, M.J., Gillen, M.F., *ibid.*, **1984**, *25*, 1669-1672.

62. Pon, R.T., Ogilvie, K.K., *ibid.*, 713-716.

63. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zim, K., Green, M.R., *Nucleic Acids Res.*, **1984**, *12*, 7035-7056.

64. a. Chamberlin, M., Ring, J., *J. Biol. Chem.*, **1973**, *248*, 2235-2244.

b. *Idem.*, *ibid.*, 2245-2250.

65. Buttler, E.T., Chamberlin, M.J., *ibid.*, **1982**, *257*, 5772-5778.

66. Lynn, D.A., Angerer, L.M., Bruskin, A.M., Klein, W.H., Angerer, R.C., *Proc. Natl. Acad. Sci. USA*, **1983**, *80*, 2656-2660.

67. DeLeon, D.V., Cox, K.H., Angerer, L.M., Angerer, R.C., *Devel. Biol.*, **1983**, *100*, 197-206.

68. Cox, K.H., DeLeon, D.V., Angerer, L.M., Angerer, R.C., *ibid.*, **1984**, *101*, 485-502.

69. England, T.E., Bruce, A.G., Uhlenbeck, O.C., *Methods Enzymol.*, **1980**, *65*, 65-74.

70. England, T.E., Uhlenbeck, O.C., *Biochemistry*, **1978**, *17*, 2069-2076.

71. Silber, R., Malathi, V.G., Hurwitz, J., *Proc. Natl. Acad. Sci. USA*, **1972**, *69*, 3009-3013.

72. Cranston, J.W., Silber, R., Malathi, V.G., Hurwitz, J., *J. Biol. Chem.*, **1975**, *249*, 7447-7456.

73. Linne, T., Oberg, B., Philipson, L., *Eur. J. Biochem.*, **1974**, *42*, 157-165.

74. Kaufmann, G., Klein, T., Littauer, U.Z., *FEBS Lett.*, **1974**, *46*, 271-275.

75. Walker, G.C., Uhlenbeck, O.C., Belows, E., Gumport, R.I., *Proc. Natl. Acad. Sci. USA*, **1975**, *72*, 122-126.

76. Hinton, D.M., Baez, J.A., Gumport, R.I., *Biochemistry*, **1978**, *17*, 5091-5097.

77. Hinton, D.M., Gumport, R.I., *Nucleic Acids Res.*, **1979**, *7*, 453-464.

78. Snopek, T.J., Sugino, A., Agarwal, K.L., Cozzarelli, N.R., *Biochem. Biophys. Res. Commum.*, **1976**, *68*, 417-424.

79. Sugino, A., Snopek, T.J., Cozzarelli, N.R., *Fed. Proc.*, **1976**, *35*, 1377.

80. *Idem.*, *J. Biol. Chem.*, **1977**, *252*, 1732-1738.

81. Sugino, A., Goodman, H.M., Heyneker, H.L., Shine, J., Boyer, M.W., Cozzarelli, N.R., *ibid.*, 3987-3994.

82. Uhlenbeck, O.C., Gumport, R.I., in *The Enzymes*, **1982**, P.D. Boyer, ed., 3rd. edition, *15*, 31-58, Academic Press, NY.

83. Sninsky, J.J., Last, J.A., Gilham, P.T., *Nucleic Acids Res.*, **1976**, *3*, 3157-3166.

84. Ohtsuka, E., Nishikawa, S., Sugiura, M., Ikehara, M., *ibid.*, 1613-1623.

85. England, T.E., Gumport, R.I., Uhlenbeck, O.C., *Proc. Natl. Acad. Sci. USA*, **1977**, *74*, 4839-4842.

86. Kikuchi, Y., Hishinuma, F., Sakaguchi, K., *ibid.*, **1978**, *75*, 1270-1273.

87. Romaniuk, E., McLaughlin, L.W., Neilson, T., Romaniuk, P.J., *Eur. J. Biochem.*, **1982**, *125*, 639-643.

88. David, M., Veckstein, R., Kaufmann, G., *Proc. Natl. Acad. Sci. USA*, **1979**, *76*, 5430-5434.

89. Dasgupta, R., Kaesberg, P., *ibid.*, **1977**, *74*, 4900-4904.

90. Ohtsuka, E., Miyake, T., Tanaka, S., Nishikawa, S., Ikehara, M., *Nucleic Acids Res.*, **1975**, special publication, s145-149.

91. Kaufmann, G., Kallenbach, N.R., *Nature (London)*, **1975**, *254*, 452-454.

92. Kaufmann, G., Littauer, U.Z., *Proc. Natl. Acad. Sci. USA*, **1974**, *71*, 3741-3745.

93. Last, J.A., Anderson, W.F., *Arch. Biochem. Biophys.*, **1976**, 167-176.

94. Uhlenbeck, O.C., Cameron, V., *Nucleic Acids Res.*, **1977**, *4*, 85-98.

95. Krug, M., Uhlenbeck, O.C., *Biochemistry*, **1982**, *21*, 1858-1864.

96. Krug, M., de Haseth, P.L., Uhlenbeck, O.C., *ibid.*, 4713-4720.

97. Gumport, R.I., Uhlenbeck, O.C., in *Gene Amplification and Analysis, Vol. 2. Structural Analysis of Nucleic Acids*, J.G. Chirikjian and T.S. Papas, eds., 314, Elsevier/North, Holland, NY.

98. England, T.E., Uhlenbeck, O.C., *Nature (London)*, **1978**, *275*, 560-561.

99. Bruce, A.G., Uhlenbeck, O.C., *Nucleic Acids Res.*, **1978**, *5*, 3665-3677.

100. Barrio, J.R., Barrio, M.C.G., Leonard, N.J., England, T.E., Uhlenbeck, O.C., *Biochemistry*, **1978**, *17*, 2077-2081.

101. Honjo, M., Imai, K., Furukawa, Y., Morijama, H., Yasumatsu, K., Imada, A., *Takeda Kenyuskko Nempo*, **1963**, *22*, 47.

102. Imai, K., Fujii, S., Takanohashi, K., Furukawa, Y., Masuda, T., Honjo, M., *J. Org. Chem.*, **1969**, *34*, 1547-1550.

103. Simoncsits, A., Tomasz, J., *Biochim. Biophys. Acta*, **1974**, *340*, 509-515.

104. Simoncsits, A., Tomasz, J., Allende, J.E., *Nucleic Acids Res.*, **1975**, *2*, 257-263.

105. Hall, R.H., Khorana, H.G., *J. Am. Chem. Soc.*, **1955**, *77*, 1871-1875.

106. Yoshikawa, M., Sakuraba, M., Kusashio, K., *Bull. Chem. Soc. Jpn.*, **1970**, *43*, 456-461.

107. Kornberg, A., Pricer, W.E., *J. Biol. Chem.*, **1950**, *186*, 557-567.

108. Wang, T.P., Shuster, L., Kaplan, N.O., *ibid.*, **1954**, *206*, 299-309.

109. Bannikova, G.E., Varlamov, V.P., Rogozhin, S.V., *Bioorg. Khim.*, **1978**, *4*, 825-831.

110. Richards, G.M., Laskowski, Sr., M., *Biochemistry*, **1969**, *8*, 4858-4865.

111. Richardson, C.C., *Proced. Nucleic Acids Res.*, **1971**, *2*, 815-828.

112. Bergstrom, D.E., Ruth, J.L., *J. Carbohyd. Nucleosides Nucleotides*, **1977**, *4*, 257-269.

113. Bergstrom, D.E., Ogawa, M.K., *J. Am. Chem. Soc.*, **1978**, *100*, 8106-8112.

114. Heck, R.F., *J. Am. Chem. Soc.*, **1968**, *90*, 5518-5526.

115. Hakimelahi, G.H., Proba, Z.A., Ogilvie, K.K., *Can. J. Chem.*, **1982**, *60*, 1106-1113.

116. Stawinski, J., Hozumi, T., Narang, S.A., Bahl, C.P., Wu, R., *Nucleic Acids Res.*, **1977**, *4*, 353-371.

117. Yoshikawa, M., Kato, T., Takenashi, T., *Bull. Chem. Soc. Jpn.*, **1969**, *42*, 3505-3508.

118. Sowa, T., Ouchi, S., *ibid.*, **1975**, *48*, 2084-2090.

119. Simoncsits, A., Tomasz, J., *Biochim. Biophys. Acta*, **1975**, *395*, 74-79.

120. Tomasz, J., Simoncsits, A., *J. Carbohyd. Nucleosides Nucleotides*, **1975**, *2*, 315.

121. Hata, T., Yamaguchi, K., Honda, S., Nakagawa, I., *Chem. Lett.*, **1978**, 507-508.

122. Honda, S., Urakami, K., Terada, K., Sato, Y., Kohno, K., Sekine, M., Hata, T., *Tetrahedron*, **1984**, *40*, 153-163.

123. Sekine, M., Matsuzaki, J., Hata, T., *Tetrahedron Lett.*, **1981**, *22*, 3209-3212.

124. Perrin, D.D., Armarego, W.L.F., Perrin, D.R., *Purification of Laboratory Chemicals*, **1966**, 325, Pergamon Press Ltd., NY.

125. Ogilvie, K.K., Beaucage, S.L., Schifman, A.L., Theriault, N.Y., Sadana, K.L., *Can. J. Chem.*, **1978**, *56*, 2768-2780.

126. Weygand, F., Hoffmann, D., Wunsch, E., *Z. Naturforsch., Teil B*, **1966**, *21*, 426-428.

127. Hayes, N.V., Branch, G.E.K., *J. Am. Chem. Soc.*, **1943**, *65*, 1555-1564.

128. Cornelisse, J., Havinga, E., *Chem. Rev.*, **1975**, *75*, 353-388.

129. Cornelisse, J., de Gunst, G.P., Havinga, E., *Adv. Phys. Org. Chem.*, **1975**, *11*, 225-266.

130. Cornelisse, J., Lodder, G., Havinga, E., *Rev. Chem. Intermed.*, **1979**, *2*, 231-265.

131. Cervello, J., Figueredo, M., Marquet, J., Moreno-Manas, M., Bertran, J., Lluch, J.M., *Tetrahedron Lett.*, **1984**, *25*, 4147-4150.

132. Jelenc, P.C., Cantor, C.R., Simon, S.R., *Proc. Natl. Acad. Sci. USA*, **1978**, *75*, 3564-3568.

133. Castello, A., Marquet, J., Moreno-Manas, M., Sirera, X., *Tetrahedron Lett.*, **1985**, *26*, 2489-2492.

134. Iverson, B., Sutter, M., unpublished research.

135. Iverson, B., unpublished research.

136. Sutter, M., Iverson, B., unpublished research.

137. Greenhut, J., Rudolph, F.B., *J. Chromatogr.*, **1985**, *319*, 461-466.

138. Maniatis, T., Fritsch, E.F., Sambrook, J., *Molecular Cloning: A Laboratory Manual*, **1982**, 250-251, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

139. Tanaka, T., Weisblum, B., *J. Bacteriol.*, **1974**, *121*, 354-362.

140. Maniatis *et al.*, 104-106.

141. *Ibid.*, 113-114.

142. *Ibid.*, 237-238.

143. Meares, C., personal communication.

144. Maniatis *et al.*, 451.